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Impact of Obesity on MMTV-Wnt-1 Mammary Cancer: Role of The Insulin-Like Growth Factor-1 (IGF-1)/Akt/mTOR Pathway

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by

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Dedication

I dedicate this dissertation to God, with whom all things are possible. I also dedicate it to my wonderful husband, Edwin, who has already been through this process and provided me practical dissertation help, general inspiration, and support. Finally, I dedicate this dissertation to my loving parents, who have encouraged and helped me through many years of education; may they see it as the conclusion of my formal schooling and the beginning of a lifetime of learning.

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Impact of Obesity on MMTV-Wnt-1 Mammary Cancer: Role of The Insulin-Like Growth Factor-1 (IGF-1)/Akt/mTOR Pathway

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Supervisor: Stephen D. Hursting

Obesity increases breast cancer risk and progression in postmenopausal women. The Akt/mTOR signaling pathway is activated in tumors in response to increased levels of obesity-related growth factors, including insulin-like growth factor (IGF)-1. Hence, we evaluated energy balance modulation as a mechanism for breast cancer prevention through modulation of Akt/mTOR. Studies suggest that dietary calcium can decrease weight gain, although an exact mechanism is not yet identified. Therefore, we investigated the effects of low-fat (10 kcal % fat) or high-fat (45 kcal % fat) diets containing either calcium phosphate (dairy) or calcium carbonate (supplement) on body weight in ovariectomized (OVX) C57BL/6 mice to determine if dietary calcium could overcome the effects of a high-fat diet. We showed that dairy decreased body weight, with no effect on food consumption. However, it is not known if restoration of normal weight can reverse mammary tumor progression and/or Akt/mTOR pathway activation.

To evaluate this, mice were fed a control diet, a calorie restricted regimen, or a diet-induced obesity (DIO) regimen for 17 weeks, after which the DIO mice were switched to the control diet, and this resulted in a 20% weight loss and mice of equal weight to control mice. MMTV-Wnt-1 mammary tumor cells were orthopically injected at week 20, following weight loss. At week 22, mice began placebo or RAD001, an mTOR inhibitor, treatment by oral gavage. Tumor growth and Akt/mTOR signaling were enhanced in formerly obese mice, despite reduction in weight, adiposity and serum hormone levels. RAD001 decreased tumor growth in the CR and control group, but was less effective in the formerly obese mice. In an additional study, we added a DIO gourp which was not switched to the control diet, and found that circulating IGF-1 levels remained significantly elevated in formerly obese mice relative to control and were comparable to levels in the DIO mice. We found that the mechanism of tumor progression was through enhanced Akt/mTOR signaling in both obese and formerly obese mice.

Based on the Akt/mTOR activation in MMTV-Wnt-1 tumor growth and progression, we next investigated the anticancer effects of ursolic acid (UA), a pentacyclic triterpene. It was previously shown that UA can affect Akt signaling. Our results showed that UA was effective decreasing tumor growth and Akt/mTOR signaling. Taken together, our findings show that the growth-enhancing effects of obesity on mammary tumor may persist even after weight loss and suggest that a combination of dietary and pharmacologic interventions targeting IGF-1/Akt/mTOR may be an effective strategy in the treatment of postmenopausal breast cancer.

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Chapter 1: Introduction

1.1 The obesity epidemic

During the past three decades obesity has dramatically increased among US adults and consequences of obesity have become a major public health concern (1). In most cases, obesity is the result of inadequate energy balance, in which the amount of energy in exceeds the amount of energy out. Body Mass Index (BMI) is usually utilized as an index to measure adiposity. BMI is calculated by dividing the weight (Kg) by the square of the height (m^2) of an individual (2) (accessed 08-17-09) and it is strongly correlated with disease risk. Although BMI correlates with body fat, it does not directly measure percentage of body fat, an independent predictor of body weight (2).

Obesity is associated with increased risk of several chronic diseases that include metabolic syndrome, type-2 diabetes mellitus, cardiovascular disease, and cancer (3-5).

The prevalence of obesity varies by age and sex, with some minority women populations showing an even higher risk (6). According to the Center for Disease Control and Prevention, obesity prevalence was as high as 70.4% among black women (7).

Data from national estimates of obesity in adults, as reported in the National Health and Nutrition Examination Survey (NHANES III), showed that obesity prevalence increased from 1999-2009 among adults (BMI of 34.5 Kg/m^2) (8). Also, overall prevalence of obesity in women was found to be 35.5% (9), with prevalence of overweight and obese being 70% among 60 year old women and older (10). However, the most recent report did not show an increase from 2007-2008. Currently, 34% of all adults in the US are obese and 35.5% of those are women (9).

Women with breast cancer who are overweight or gain weight after diagnosis have a poorer prognosis and higher risk of death compared with lean women (11). Women with breast cancer in the highest quartile of BMI are 2.5 times as likely to die of their disease within 5 years of diagnosis compared with women in the lowest quartile of BMI as shown in a study of 1177 premenopausal women (12). Given the high rates of obesity in women, and its correlation with chronic disease, it is critical to develop initiatives to prevent or minimize the impact of obesity on public health.

1.2 Breast Cancer in the United States

According to the American Cancer Society (ACS), Breast cancer is the second leading cause of noncutaneous cancer deaths among American women, exceeded only by lung cancer, with a higher rate of diagnosis after menopause. Incidence rates have been decreasing since 1999, which reflects a combination of factors including decreased use of hormonal replacement therapy (HRT) among postmenopausal women and delayed diagnosis as a result of decreased mammography utilization (13). Incidence rates are higher among White women while mortality rates are higher among African-American women (13).

It has been shown that obesity is a major contributor to breast cancer risk (14). The mechanisms underlying the obesity-breast cancer link are complex and include obesity effects on tumor growth and progression, therapy response, metastasis, and survival.

1.3 Obesity and Postmenopausal Breast Cancer

Epidemiological data have shown that overweight and obesity are risk factors for postmenopausal breast cancer, particularly hormone-responsive tumors (15, 16). Given the

increasing rates of obesity in women, it is critical to understand the physiological mechanisms that impact tumorigenesis. Women with breast cancer who are overweight or gain weight after diagnosis have a poorer prognosis and higher risk of death compared with lean women (17, 18). Several large-scale cohort studies such as the Cancer Prevention Study II have also confirmed this association between obesity and increased mortality from breast cancer (15).

Adipose tissue functions as a secretory organ affecting the release of many hormones, hormone-like proteins, and adipokines into circulation that regulate metabolism, inflammation, and cancer susceptibility (19). Obesity has been associated with increased insulin, estrogen, and growth factor signaling, all of which are likely to stimulate tumorigenesis (20). However, studies using the A-Zip/F-1 mouse, which lack white adipose tissue but still show accelerated tumor formation, suggest that adipokines are not required for tumorigenesis. Instead, high levels of insulin and growth factors may be responsible for activation of growth factor signaling pathways and promote cell survival and proliferation. Insulin and growth factors can also activate estrogen receptor (ER) signaling and contribute to breast cancer (21). Recent studies have demonstrated that the crosstalk between ER and growth factor signaling results in activation of PI3K/Akt signaling. This enhanced signaling has been implicated in decreased responses to multiple therapeutic modalities (22). Several hypotheses have been proposed, nevertheless, the underlying mechanism between elevated growth factor levels associated with obesity and postmenopausal breast cancer is not fully understood. Research evaluating this mechanism is greatly needed in order to design treatments to prevent the impact of obesity on postmenopausal breast cancer risk.

Given the high rates of obesity in women, it is critical to understand the physiological mechanisms that impact tumorigenesis. Besides excess adipose tissue, obesity has been

associated with increased insulin, estrogen and growth factor signaling, all of which are likely to stimulate tumorigenesis (23).

1.3.1 LEPTIN. Leptin is one of the most studied adipocyte-derived hormones. Circulating levels of leptin are directly proportional to the amount of adipose tissue and nutritional status. Therefore leptin levels are increased during obesity (24) and are thus decreased by caloric restriction. Furthermore, alterations in leptin metabolism or function can result in obesity, diabetes and imbalance in energy homeostasis (25). Leptin is involved in several biological processes including food intake regulation, immune function, and sexual development (26). Leptin expression can be regulated by insulin and in some cases by IGF-1. *In vitro*, leptin induces cell proliferation in both normal and cancerous cells. Saxena et al., showed that co-treatment with IGF-1 and leptin increased proliferation of breast cancer cells as a consequence of a bidirectional cross-talk between leptin and IGF-1 signaling (27), suggesting that elevated levels of these factors during obesity can contribute to increased breast cancer cell growth.

Since leptin levels are increased during obesity and obesity is a risk factor for postmenopausal breast cancer, several studies have assessed the mechanisms by which leptin may affect breast cancer growth. There are several signaling pathways that respond to leptin's proliferative effects making the study of leptin very complex.

Leptin exerts its growth promoting effects on the cell through activation of the JAK/STAT3, ERK1/2, and/or PI3K/akt signaling pathways (24). In addition, leptin has been shown to affect the growth of estrogen receptor positive (ER+) breast cancer cells by regulating aromatase expression and estrogen synthesis. The resultant estrogen signaling facilitates growth factor activation of key downstream signaling pathways involved in angiogenesis by increased expression of vascular endothelial growth factor (VEGF) (24). Furthermore, leptin can directly

upregulate ER even in the absence of the ligand (28) and recent findings suggest a more direct role of leptin activating the mammalian target of rapamacyn (mTOR) signaling pathway downstream of Akt (29).

1.3.2 INSULIN. Obesity leads to the body's altered response to insulin that results in a correlation between circulating insulin levels and BMI; increased insulin levels during obesity is believed to promote cancer growth and is also an adverse prognostic factor for the disease (30). Insulin activates the insulin receptor (IR) triggering a cascade of events that ultimately leads to activation of the extracellular-signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K/Akt) (31).

Epidemiological studies associate increased insulin levels and insulin resistance with increased risk of breast cancers (30). Insulin levels in type 1 diabetes patients correlate with serum IGF-1; the insulin-cancer hypothesis associates the hyperinsulinemia during obesity with decreased concentrations of insulin-like growth factor binding protein 1 (IGFBP-1) and IGFBP-2 that result in elevated levels of bioavailable IGF-1 favoring tumor growth (30, 32),. These binding proteins sequester and inhibit the action of IGF-1 (23, 33). In spite of the pro-tumorigenic and anti-apoptotic effects of IGF-1, its effects on breast cancer growth are still subject to debate. Therefore, chronic hyperinsulinemia during obesity can contribute to enhanced cell proliferation by regulating bioavailable IGF-1 through serum levels of IGFBP-1/2.

1.3.3 METABOLIC SYNDROME. The metabolic syndrome, also known as insulin resistance syndrome, is a combination of several conditions characterized by central obesity, hyperinsulinemia, increased blood pressure, and dyslipidemia; all of which increase the risk of type 2 diabetes (**Figure 1**) (5, 34). Hyperinsulinemia and insulin resistance are associated with increased risk of breast cancer, particularly postmenopausal. Besides metabolic functions, insulin

can induce cell proliferation through the insulin-like growth factor-1 (IGF-1) receptor; indicating an interrelationship between insulin signaling and cell proliferation (35).

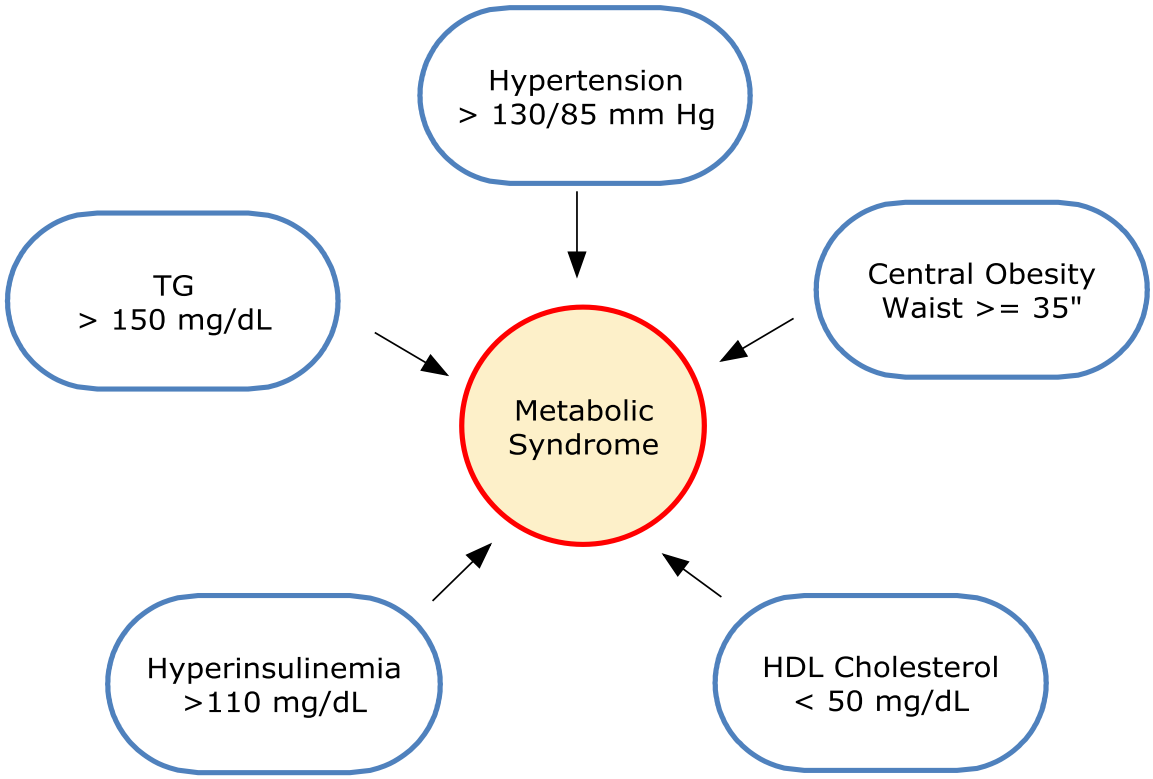

Figure 1.1. Metabolic Syndrome in Humans.

Obesity and insulin resistance are major contributing factors in the development of metabolic syndrome. A combination of conditions that include: hypertension, central obesity (waist ≥ 35", in women), low high-density lipoprotein (HDL) levels, hyperinsulinemia, and high triglycerides (TG) are used to diagnose metabolic syndrome.

1.3.4 INSULIN-LIKE GROWTH FACTOR-1 (IGF-1). IGF-1 has been shown to stimulate cell cycle progression from G1 to S phase in many normal and cancer cells, this effect is mediated in part by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and modulation of cyclin-dependent kinases (36, 37). IGF-1 production is regulated by growth hormone (GH). Obesity impairs GH secretion which is completely reversed by body weight loss. However, the relationship of IGF-1 and GH is not directly proportional (38). Over the last two decades studies looking at IGF-1 serum levels in obese individuals have yielded conflicting results and have been thought to be dependent on the type and degree of obesity (39-41). Recent studies have shown that obese subjects relative to lean, have increased "free" serum IGF-1 levels, or bioactive IGF-1, that results from a decreased production of IGFBP-1 and IGFBP-2. Most studies show IGF-1 to directly stimulate premenopausal breast cancer growth, with less clear evidence for postmenopausal breast cancer risk (42). The association between IGF-1 and postmenopausal breast cancer risk is very complex and seems to be associated with elevated IGF-1 before menopause, particularly during early life (43).

1.3.5 ESTROGEN. It is well established that circulating estrogen levels can affect breast cancer risk, particularly postmenopausal. The Nurses Health Study further supported that elevated circulating estrogen levels increase the risk of postmenopausal breast cancer, particularly hormone receptor positive cancers (44, 45).

Obese women have 35% higher concentrations of estrogen and 130% higher concentrations of estradiol compared with nonobese women (46). The current hypothesis for the correlation between obesity and breast cancer indicates that obese breast cancer patients have higher bioavailable concentrations of estrogen, which contributes to molecular crosstalk and activation of signaling pathways that increase breast cancer risk and poor survival (47).

Estrogen biosynthesis is catalyzed by a microsomal member of the cytochrome P450 superfamily (aromatase cytochrome P450) and is the product of the CYP19 gene. P450 is the sole member of family 19 (48). Estrogens are produced primarily from the synthesis of androstenedione from cholesterol in developing ovarian follicles (49). After menopause, estrogen biosynthesis results from aromatase activity in peripheral tissues, including adipose tissue, converting androgens into estrone. The circulating estrogen level is an indirect marker of estrogen produced in extragonadal sites that escapes to circulation (50). An elevated circulating estrogen level in postmenopausal women, resulting from the peripheral aromatization of androgens into estradiol by the action of aromatase, is the reflection rather than indicator of estrogen action (48). It has also been reported that inflammatory adipokines including TNF-α and IL-6, can act either in an autocrine or paracrine way to increase aromatase production, resulting in increased estrogen biosynthesis (51).

Estrogen-induced proliferation signals through the estrogen receptor alpha (ER-α), while the ER-β acts as a tumor suppressor in many cases (52). Breast tumors can be categorized as estrogen receptor positive (ER+) or negative (ER-) based on the presence of the estrogen receptor (ER) alpha.

Nuclear initiated steroid signaling (NISS). Genomic estrogen signaling, also known as canonical estrogen signaling, is initiated by estrogen binding to its nuclear receptor. In the absence of estrogen, heat shock proteins (Hsp) bind to the ER and render it inactive. Upon ligand binding, ERs dissociate from Hsp, dimerize, and translocate to the nucleus where the dimer binds to genes comprised of estrogen response elements (ERE). This binding results in activation of gene transcription, of both gene activators and inhibitors, dependent on cofactor recruitment (53). In the presence of estrogen, association with ER occurs and initiates a multiple cascade of

events that lead to expression of genes involved in cell function regulation (52). This is known as genomic activity and is known to regulate the transcription of genes important for cell growth in most breast tumors (i.e. IGF-1R, PR, cyclin D1). In addition to regulating transcription of ERE containing genes, ER can bind other transcription factors such as AP- and SP-1 (53).

Membrane initiated steroid signaling (MISS). Recent evidence shows that a fraction of ERs can also be localized in the plasma membrane and are able to dimerize. Once hormone binding to the receptor occurs it triggers a number of events resulting in gene transcription (54). Evidence shows that ER-regulated gene transcription is not completely dependent on estrogen binding to its receptor (53). It has become evident that the contribution of estrogen signaling to breast cancer is more complex than anticipated due to the membrane-associated (non-genomic) ER signaling (53). The non-genomic ER signaling (also known as membrane initiated steroid signaling (MISS) (**Figure 2**), results when membrane-bound ERs interact with cytosolic estrogen resulting in crosstalk with growth factor signaling pathways leading to PI3K/Akt activation as previously noted. This promotes cell cycle progression and inhibition of apoptosis, both known to affect cancer risk and response to therapy (53). In obese postmenopausal patients, elevated estrogen synthesis from peripheral tissues, including adipose, is known to modify response to therapy and overall survival (55, 56) . However, no studies have evaluated the nongenomic ER signaling in obese postmenopausal breast cancer patients.

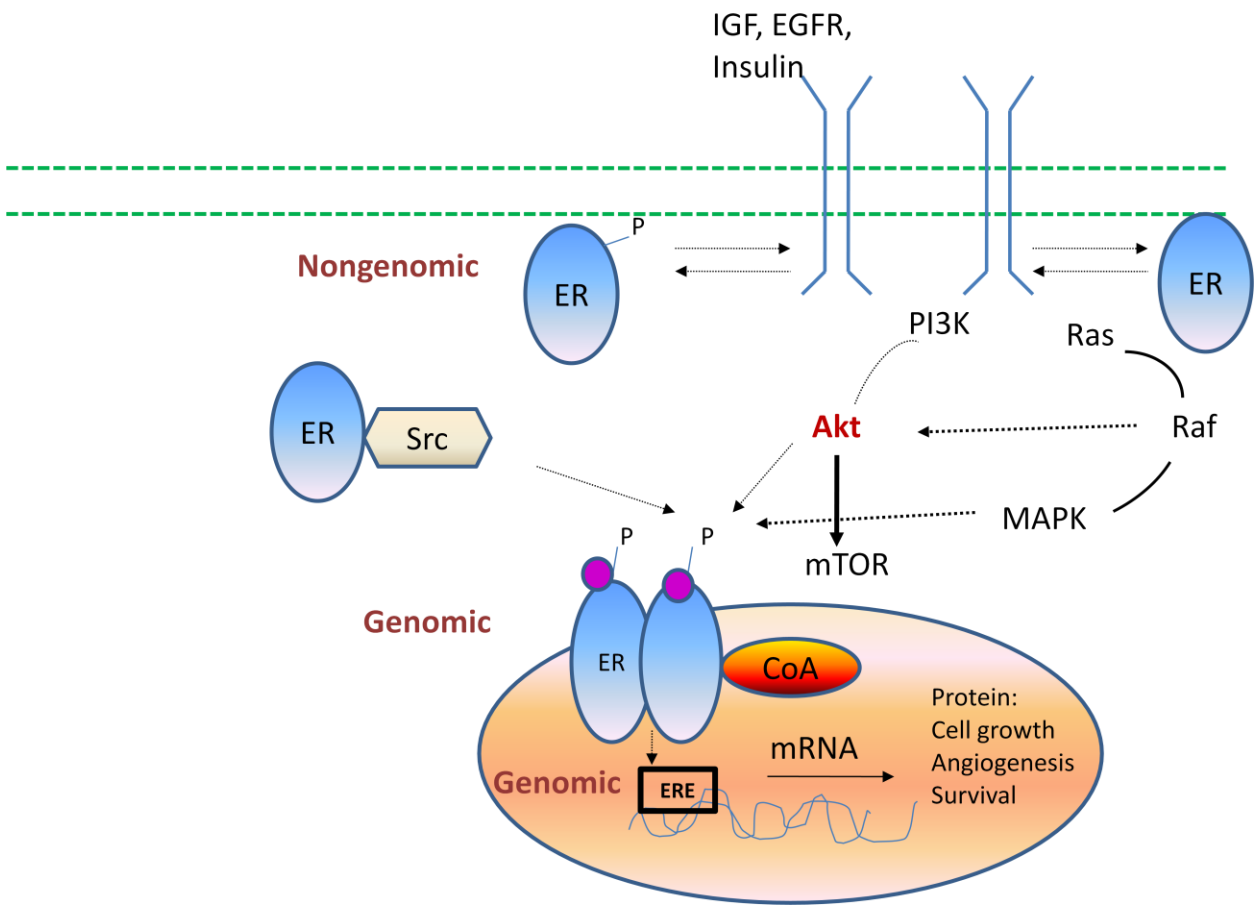

Figure 1.2. Genomic and nongenomic estrogen signaling.

In most ER-positive tumors, nuclear initiated steroid signaling predominates and is characterized by estrogen receptor (ER) action as a transcription factor in the nucleus. Membrane in initiated steroid signaling can also occur. This is mediated by interaction of ER in the plasma membrane or the cytoplasm with various growth factor receptor and cellular kinase signaling molecules.

1.4 Obesity reversal and breast cancer risk

Obesity and weight gain are well established risk factors for postmenopausal breast cancer (44). However, the definite role of weight loss on postmenopausal breast cancer risk remains to be fully elucidated.

In addition to decreased estrogen levels, weight loss has also been associated with beneficial effects on co-morbid conditions, such as diabetes, insulin resistance, and hypertension (11, 57). Considering that 2/3 of American women over the age of 50 are overweight or obese (10), weight loss could bring tremendous health benefits including a decrease in breast cancer risk. Studies evaluating the effect of weight loss on breast cancer patients are limited and have mixed results (11). Kotsopoulos et al., showed that premenopausal weight loss (between 30-40 years of age) protects against breast cancer between development in women carriers of either BRCA1 or BCRA2 mutations (58).

Studies from our laboratory have shown that formerly obese ovariectomized mice display increased tumor growth in spite of weight loss when compared to never obese control mice (unpublished data). This effect is puzzling considering that epidemiological studies have shown that a decrease in fat intake is able to decrease endogenous estrogen in both premenopausal and postmenopausal women (11). Preclinical and clinical studies are needed in order to understand the effects postmenopausal weight reduction on circulating hormones and breast cancer risk.

1.5 Endocrine Therapy for Breast Cancer

Since estrogen and its receptor play a central role in ER-α (+) breast cancers, they are usual targets for therapy. After menopause, aromatase is the enzyme responsible for converting androgens into estrogen in peripheral tissues and adipose (59). Aromatase activity is known to

be increased with age, and obese postmenopausal patients have high levels of aromatase that correlate with body mass index (BMI) (59). Since most breast tumors are ER-α (+) and therefore respond to the mitogenic effects of estrogen, elevated circulating estrogen and aromatase levels in obese postmenopausal women are associated with poor survival, and this is believed to be involved in the link between BMI and cancer risk (60). The antiestrogen tamoxifen has been the standard treatment for estrogen-responsive postmenopausal breast cancer patients. It acts by blocking estrogen binding to the ER. However, the development of tamoxifen resistance and other complications decrease the effectiveness of tamoxifen for these patients.

Due to the contribution of aromatization to breast cancer, a number of aromatase inhibitors (AIs) have been developed for treating postmenopausal breast cancer patients. This type of drug binds reversibly (nonsteroidal) or irreversibly (steroidal) to the aromatase enzyme, thereby decreasing estrogen synthesis. Preclinical and clinical studies have demonstrated the superiority of AIs, as compared to tamoxifen, for the treatment of postmenopausal breast cancer and are now used as a first line of therapy for those cases (60-63). AIs are also superior as both adjuvant and neoadjuvant treatment and have reduced estrogenic effects compared with tamoxifen (63). An example is anastrozole (registered as Arimidex), a potent nonsteroidal AI currently used as a first line therapy for postmenopausal breast cancer patients. However, few studies have addressed interactions among adjuvant therapy, obesity, and clinical outcomes and no published clinical studies have evaluated the efficacy of AIs on obese patients.

1.6 Resistance to Endocrine Therapy

Development of therapy resistance is a major impediment for breast cancer therapeutics. About half of the cases will develop *de novo* or acquired resistance (63). Resistance to endocrine

therapy is believed to be the consequence of hyperactivation of signaling pathways that are utilized by breast cancer cells to bypass normal endocrine dependence (64). Therapy directed to target several signaling pathways at different nodal points is a potential mechanism to eliminate molecular crosstalk responsible for decreased therapeutic response. Among other factors, the activation of Akt/mTOR plays a critical role in resistance to therapy, as it is a key adaptive change driving endocrine resistance (65). Once activated, Akt induces the assembly of the rapamycin-sensitive complex (Raptor), also known as mTORC1, of the mammalian target of rapamycin (mTOR). In recent years there has been an increased rationale for effective co-treatment with drugs targeting aberrant signal transduction pathways in patients receiving endocrine therapy, particularly since long-term estrogen deprivation results in therapy resistance in most cases (63). Several studies have demonstrated that endocrine therapy, in parallel with small molecule signal transduction inhibitors (STIs) of mTOR can inhibit tumor cell proliferation and restore response to endocrine therapy both *in vivo* and *in vitro* (66-69). This new strategy seems to improve efficacy of endocrine therapy agents and is contributing to the decline of endocrine resistance in many hormone-sensitive breast cancers. However, it is not known how obesity will affect the efficacy of these agents alone or in combination.

1.7 Akt/mTOR Signaling

Akt is a serine/threonine kinase downstream of PI3K usually activated in several human cancers. Increased Akt activation is associated with resistance to apoptosis and increased cell proliferation and survival (reviewed in (70)), including breast cancer. Akt is also activated in the obese-insulin resistance state by growth factors such as IGF-1 (71). Akt activation plays a critical role in drug resistance and is believed to promote proliferation and increased cell survival thereby

contributing to cancer progression. mTOR is a critical downstream effector of Akt responsible for regulating cell function and its activation is also implicated in tumorigenesis and development of resistance to therapy (72). Previous studies have demonstrated that Akt-induced resistance to chemotherapeutic agents can be reversed through parallel administration of mTOR inhibitors (67, 73). Kirkegaard et al., (74) demonstrated that Akt/mTOR activation mediates increased resistance to endocrine therapy and death (hazard ratio 3.22) among patients treated with tamoxifen for ER+ breast cancer. The development of postmenopausal breast cancer has consistently been associated with obesity and activation of signaling pathways such as Akt and mTOR (**Figure 3**). However, it is still unclear whether activation of the Akt/mTOR pathway is also increased during elevated growth factor signaling associated with obesity and if it could be involved in the promotion of therapy resistance. In addition to Akt activation by IGF-1, experimental evidence suggests that obesity may also activate downstream targets of Akt. Khamzina et al. (75) have shown that basal (fasting state) activation of mTOR and its downstream target S6K1 is markedly elevated in liver and skeletal muscle of obese rats fed a high fat diet compared with chow-fed, lean controls. Time-course studies also revealed that mTOR and S6K1 activation by insulin was accelerated in tissues from obese rats. This suggests that obesity, probably through elevated growth factor signaling, influences the PI3K/Akt/mTOR pathway at many levels, but it is still unclear how this effects breast tumor response. Furthermore, leptin induced phosphorylation of P70S6K and 4EBP1 in macrophages *in vitro* and this was prevented by inhibition of either PI3K or mTOR signaling with LY294002 or rapamycin, respectively (76). Since the effects of leptin on mTOR signaling are sensitive to rapamycin, it implies that TORC1 complex activation may be involved, and that increased leptin levels in the obese state will directly contribute to mTOR activation.

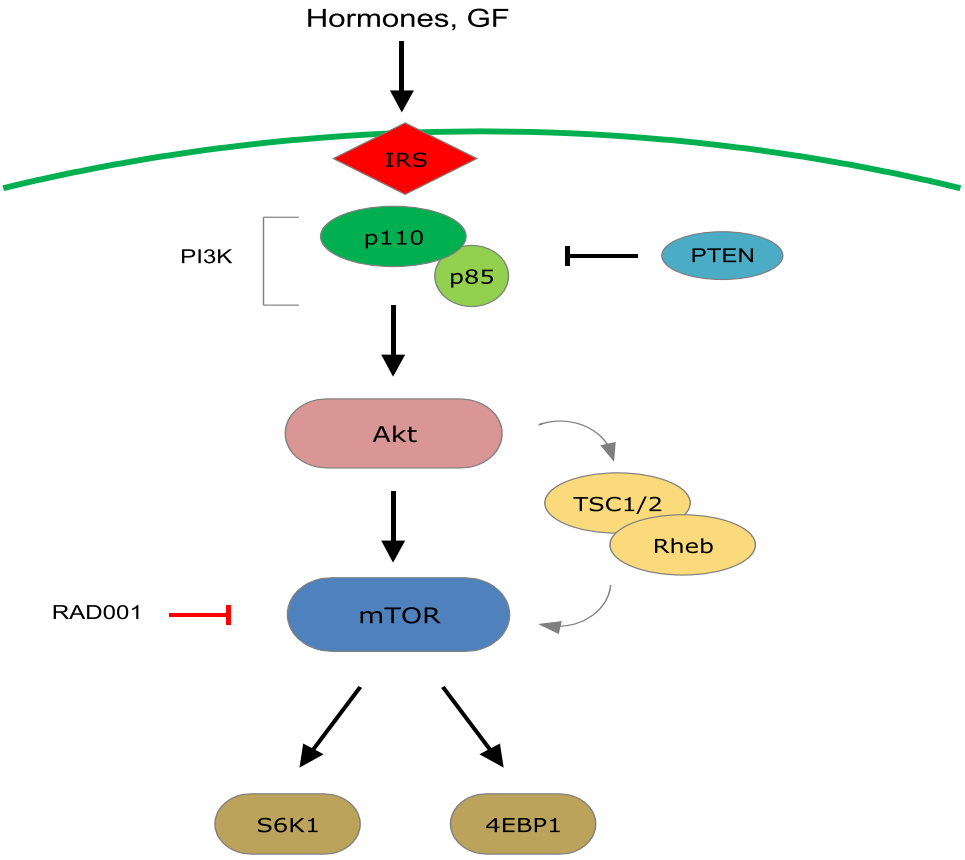

Figure 1.3. Akt/mTOR pathway is critical for the proliferative responses of breast cancer cells

Homone and growth factor binding activates the IR (Insulin Receptor) tyrosine kinase which phosphorylates IRS1 or IRS2. PI3K (p110 catalytic subunit, p85 regulatory subunit) binds phosphorylated IRS, resulting in activation of Akt. Akt phosphorylates mTOR directly, or through TSC2, and activates the translation of proteins that regulate cell survival and proliferation.

1.7.1 mTOR Complexes

The mammalian target of rapamycin (mTOR) is a 289 Kda serine/threonine protein kinase that acts as a major effector regulating cell growth and metabolism in response to nutrient signals (77). mTOR forms complexes with other proteins, such as rictor and raptor, which are involved in mediating signaling downstream of mTOR and participate in mTOR complex 1 (mTORC1) and mTORC2 complexes (78). mTOR inhibitors, including rapamycin and its derivatives, act by forming an inhibitory complex with FK506 binding protein (FKBP-12) an intracellular receptor that binds mTORC1.

1.7.2 mTOR Inhibition

mTOR is a member of the phosphatidylinositol 3-kinase (PI3-K)-related family of kinases that regulate multiple processes including, cell cycle progression and metabolism (79). mTOR binds to the cytoplasmic receptor FKBP-12 resulting in initiation of downstream signaling events (80). Rapamycin and its derivatives, bind with high affinity to FKBP-12 resulting in mTOR inhibition and decreased phosphorylation of two downstream targets of mTOR, p70 S6 kinase and 4E-BP1. These events inhibit translation of critical mRNAs involved in cell cycle progression and cell proliferation (80). mTOR inhibition has proven to be a good strategy in hormone receptor positive breast cancers and in most cases, mTOR inhibition correlates with activation of Akt (81).

1.7.3 mTOR Inhibition and Lipid Metabolism

mTOR is a well conserved serine/threonine kinase that integrates nutrient and growth factor signals resulting in regulation of cell growth and protein synthesis (77). mTOR-regulated mRNA translation and protein synthesis are two processes that consume cellular energy and are

known to respond to insulin action (77). mTOR has been shown to play a key role in lipid metabolism. mTOR expression has been shown to dramatically increase in differentiated adipocytes (82) and rapamycin inhibits adipocytes differentiation (83). However, the exact mechanism by which mTOR regulates energy metabolism is not yet fully understood. When glucose levels are increased, the liver plays a major role in *de novo* lipid synthesis, resulting in further stimulation of insulin secretion by the pancreatic β cells, causing insulin resistance (84). In addition, mTOR activation has been observed in the liver of high-fat-fed obese rats (75), suggesting that mTOR is a likely target for obesity-related cancers.

1.8 Signal Transduction Inhibitors and Breast Cancer

Resistance to endocrine therapy is believed to be the consequence of hyperactivation of signaling pathways that are utilized by breast cancer cells to bypass normal endocrine dependence (64). Therapy directed to target several signaling pathways at different nodal points is a potential mechanism to eliminate molecular crosstalk that is responsible for decreased response to therapy. Clark et al., (85) demonstrated *in vitro* that tamoxifen treatment increases activation of Akt in hormone-dependent breast cancer cells and that tumor cell death was increased when chemotherapy-induced apoptosis was combined with the PI3K inhibitor LY294002. This finding confirms that multiple signaling pathways can be overexpressed in breast cancer. Based on the potential involvement of multiple signaling pathways in the development and progression of breast cancer, combination of STIs with endocrine therapy may be a potential approach to circumvent endocrine resistance.

In recent years there has been an increased rationale for effective co-treatment with drugs targeting aberrant signal transduction pathways in patients receiving endocrine therapy,

particularly since long-term estrogen deprivation results in therapy resistance in most cases (63). RAD001 (Everolimus) is an orally bioavailable STI agent that targets and inhibits the mTOR signaling pathway. Increasing *in vitro* evidence has led to several clinical trials designed to test the effectiveness of endocrine therapy combined with STIs at the clinical level. Combination of the AI letrozole and RAD001 has been investigated in human breast cancer cells (66) and results from this study indicate that targeting mTOR with RAD001 can potentially prevent the development of letrozole resistance. This new strategy seems to improve efficacy of endocrine therapeutic agents and is contributing to the decline of endocrine resistance in many hormone-sensitive breast cancers. How obesity affects the efficacy of these agents alone or in combination in not yet known .

1.9 Natural agents in the treatment of postmenopausal breast cancer.

Naturally occurring agents are an alternative for improving cancer therapeutics. Research has provided convincing evidence that diets rich in fruits and vegetables may provide protective effects against the risk of several types of cancers. This anticarcinogenic effect may be due to bioactive food components directly targeting pathways that become aberrant during cancer growth. However, the role of many bioactive food components in breast cancer is not yet fully understood. Therefore, studies investigating specific components in different cancer models are warranted to identify more effective cancer therapeutics, particularly for breast cancer.

A significant proportion of the drugs currently used to treat cancer are either natural products or compounds based on natural products (86), proving them to be an effective approach for prevention and treatment of many cancers (87, 88). Ursolic acid (UA) is a pentacyclic triterpene member of the cyclosqualenoid family, which are ubiquitous in the plant kingdom (89)

and therefore can be found in many foods and herbs, such as apple, cranberry, rosemary, and oregano. Studies have shown that UA can inhibit several cancers and improve glucose tolerance in high-fat-fed mice (90, 91). *In vitro* studies have shown UA to be an anticancer agent that acts through control of cell growth, differentiation, and apoptosis (92). Secondary metabolites of triterpenes, such as camptothecin and paclitaxel, are currently used as chemotherapeutic agents (93). Limited knowledge, however, exists of the effects of UA on major molecular pathways involved in cancer progression after menopause.

The Akt and mitogen-activated protein kinase **(**MAPK) cellular signaling pathways are important in cancer development and progression. Bioactive food components have been shown to affect these biomarkers, as well as others, both *in vitro* and *in vivo* (94-97), suggesting their use as chemopreventive and chemotherapeutic agents. It has been shown that UA can inhibit growth of endometrial cancer cells, and this effect is mediated by inhibition of both PI3K/Akt and MAPK signaling pathways (98). However, studies regarding UA effects are limited with little indication of the impact that UA may have on molecular pathways activated during postmenopausal breast cancer. Nevertheless, its anticancer effects and its low toxicity (99) warrant further study into the possible effects of UA on inhibiting mammary tumor growth in a postmenopausal model.

1.10 The Wnt-1 Model and Wnt Signaling in Breast Cancer

One significant impediment to improving our understanding of the obesity-breast cancer link is the lack of appropriate animal models for spontaneous mammary tumorigenesis. The Wnt-1 mouse model has become a highly regarded model used to study the obesity and breast cancer relationship.

The Wnt-1 transgenic (Wnt-1 TG) mouse is predisposed to the development of ER+ mammary adenocarcinomas which are responsive to tamoxifen treatment. Females who express the Wnt-1 proto-oncogene develop mammary carcinomas by 9 months of age, resulting in more rapid mammary tumor development and higher metastatic rates (~45%; liver and lung). These mice are on a C57BL/6 background, a model that has a propensity to become obese. The Wnt-1 mammary tumor cells over-express the Wnt-1 protein and are harvested directly from the spontaneous tumors of Wnt-1 TG mice.

Wnt signaling has been implicated in human breast cancer. Estrogen and tamoxifen regulated genes include the Wnt-5A family member (100). Furthermore, expression of Wnt-1 and Wnt target genes have been shown to be upregulated in human breast cancer cases (101), and Wnt-mediated mTOR activation is a critical step in mouse mammary tumor virus (MMTV)-induced tumors in mice (102). Since Wnt-induced mTOR activation is sensitive to rapamycin, treatment with mTOR inhibitors has the potential to be an effective therapy for tumors driven by the Wnt signaling pathway.

1.11 Significance

There is mounting evidence that links obesity with decreased survival and poorer prognosis of postmenopausal breast cancer patients. However, little is known about the effects of obesity on response to therapeutic agents. The existing evidence is based mainly on epidemiological studies. No study to date has focused on identifying molecular changes in Akt/mTOR signaling during obesity that may affect response to therapy. We hypothesized that mTOR inhibition with both a pharmacological agent (RAD001) and a natural compound (UA) would significantly diminish the protumorigenic effects of obesity in a postmenopausal mouse model of breast cancer.

1.12 Hypothesis

Since Akt/mTOR signaling has been implicated in breast cancer, and obesity (through activation of GF signaling pathways) can activate this pathway, we hypothesized that the growth-enhancing effects of obesity on mammary tumor may persist even after weight loss suggesting that a combination of dietary (UA) and pharmacologic interventions (RAD001) targeting IGF-1/Akt/mTOR may be an effective strategy in the treatment of postmenopausal breast cancer.

1.13 Rationale

Breast cancer is the second leading cause of death among women in the US and it is more frequently diagnosed after menopause. Since obesity is an established risk factor for breast cancer, approaches aimed to decrease weight gain are potential strategies to decrease the burden of breast cancer. The effects of obesity on breast cancer promotion and progression are mediated through complex mechanisms that include activation of molecular pathways like the Akt/mTOR signaling pathway. Effective targeting of Akt/mTOR with pharmacological drugs and/or natural components are effective strategies to decrease postmenopausal breast cancer.

We tested our hypothesis using a mouse model of postmenopausal breast cancer. We first used dietary calcium supplementation to establish the effects on body weight. We then used a diet-induced obesity mouse model to establish obesity-driven molecular changes in the Akt/mTOR signaling. We further investigated the mechanistic effects of former obesity comparing obese and

formerly obese with never obese control mice. Finally, we tested the effects of UA in a mouse model of postmenopausal breast cancer and Akt/mTOR signaling.

Chapter 2: Dietary Calcium Source Influences Body Composition, Glucose Metabolism and Hormone Levels in a Mouse Model of Postmenopausal Obesity

2.1 Abstract

Background: The prevalence of obesity has risen dramatically, with postmenopausal women particularly prone to increased adiposity. Epidemiologic data suggest that dietary calcium, particularly from dairy products, can decrease weight gain. The aim of this study was to evaluate effects of different calcium sources in a mouse model of postmenopausal obesity. Methods: Ovariectomized C57BL/6 mice were randomized to either low-fat (LF) or high-fat (HF) diets containing either calcium phosphate from non-fat dried milk and whey mineral concentrate (dairy) or calcium carbonate (supplement). Results: Dairy, but not supplement, decreased weight gain and percent body fat in HF mice, with no effect on food consumption. Dairy improved insulin resistance and glucose tolerance, while supplement increased bone mineral density in LF mice. Dairy had no effect on bone. Conclusion: The beneficial effects of dietary calcium on body weight and bone health after menopause may be significantly influenced by other dietary components.

2.2 Introduction

Obesity rates continue to increase and represent a major public health problem in the US (103). The prevalence of obesity among American women aged 20 years and older increased from 15.6% in 1960 to 34% in 2000 (103). Obesity results from significant increases in white adipose tissue, which acts as a major secretory organ that releases a number of hormones and adipokines, including leptin, adiponectin, resistin, monocyte chemoattractant protein (MCP)-1 and plasminogen activator inhibitor (PAI)-1 (104). Obesity is characterized by a chronic state of pro-inflammation (105), and the elevated production of inflammation-related adipokines is believed to be important in the development of a number of chronic diseases linked to obesity, particularly type 2 diabetes, metabolic syndrome and many cancers (104).

Although energy balance is the most critical factor in body weight regulation (106), an inverse relationship between dietary calcium and adiposity has been found in some human and animal studies (107). It has also been shown that high dietary calcium modulates energy metabolism and adipose tissue cytokine production (108). Dietary calcium is required for many biological processes (109), and calcium-rich diets are correlated with a decreased risk of disease (110). Several studies suggest that high dietary calcium decreases obesity, serum triglyceride values and insulin resistance (111, 112). In the Coronary Artery Risk Development in Young Adults (CARDIA) Study, these beneficial effects were observed in overweight, but not normal weight, subjects (113). Furthermore, several studies demonstrated that dairy-containing foods exert a substantially greater effect in weight regulation than supplemental (calcium carbonate) sources of calcium (114). In recent animal studies, a diet high in calcium from non-fat dry milk decreased body weight and fat content in male Wistar rats (115), and yogurt supplementation decreased weight gain but did not affect insulin sensitivity in male mice fed a moderate-fat diet

(116). Because dairy products are generally fatty foods, it is difficult to study the effects of dairy in the context of both low dietary fat and high dietary fat. In addition, the concentration of milk components is not always constant; whey proteins, sugar and even calcium content are known to fluctuate depending on many factors (117), which increase the challenge of simultaneously controlling calcium source and concentration together with fat content and protein composition in the diet.

Dietary calcium is also known to be critical for the development and maintenance of bone density (118). However, in some studies the effects of dairy foods *versus* supplemental calcium on bone health are inconsistent. Budek *et al.* found that, when adjusted for protein intake, high intake of dairy actually decreased markers of bone turnover (119), while the Nurses' Health Study found no protective effect of milk consumption in fracture risk (120).

These studies suggest that calcium consumed as supplemental calcium or as dairy products may have differential effects on energy metabolism and bone health. Although the risks of obesity and of osteoporosis increase after menopause (120), little is known about the effects of dietary calcium on postmenopausal obesity and metabolism. The ovariectomized C57BL/6 mouse, in which surgical removal of the ovaries mimics the loss of ovarian estrogens after menopause, is the model of choice for investigating issues related to postmenopausal calcium malabsorption (121). However, there are no animal studies examining the effect of different sources of dietary calcium, such as calcium from dietary calcium supplements (usually calcium carbonate) and calcium from dairy (mainly as calcium phosphate and in combination with other dairy components), on body composition and metabolism in an animal model of postmenopause.

In the present study the effects of two different sources of calcium (supplement and dairy) on body composition, bone mineral density and endocrine parameters are compared in a model of postmenopausal obesity.

2.3 Materials and Methods

2.3.1 Animals and Diets.

Six-week-old ovariectomized C57BL/6 mice (n=108) and a group of 12 sham-ovariectomized mice, in which surgery was performed but the ovaries were not removed, were obtained from Charles River Laboratories, Inc. (Animal Production Area, NCI-Frederick, Frederick, MD, USA) and placed on a chow diet. All mice were individually housed, consumed food and water ad libitum, and were on a 12 h light/dark cycle. One week after arrival, ovariectomized mice were randomly assigned (n=18 per group) to either a high-fat (HF, 46 kcal% fat) or a low-fat (LF, 10 kcal% fat) diet varying in calcium amount and source: control, with ~0.6% (by weight) calcium as calcium carbonate; supplement, with ~2% calcium as calcium carbonate; or dairy, providing ~2% calcium as calcium phosphate through the use of non-fat dry milk and TruCal® D50, a whey mineral concentrate (Glanbia Nutritionals, Inc., Monroe, WI, USA). All experimental diets were formulated by Research Diets, Inc. (New Brunswick, NJ, USA) and were comparable within dietary fat categories except for the amount and source of calcium and source of protein (Table I). The dietary groups were categorized as follows: 1) LF control; 2) LF supplement; 3) LF dairy; 4) HF control; 5) HF supplement; or 6) HF dairy. The sham-ovariectomized mice were fed the HF control diet for the duration of the study and served as an additional control group. All animal protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and carried out in

compliance with all guidelines and regulations. Throughout the 20-week study, mice consumed diets ad libitum; feed intake for all groups was measured three times a week and body weight was measured weekly.

At weeks 5 and 10 of the study, after a 10-hour fast with access to water ad libitum, mice were lightly anesthetized with isofluorane for collection of a 150-µL blood sample via the retro-orbital sinus. Whole blood was allowed to clot at room temperature for 30 min prior to centrifugation at 1000 x g for 10 min, and the serum was stored at -80 °C for analysis.

After euthanasia, carcasses were stored at -20 °C. Fat weight, lean weight, bone mineral density (BMD) and bone mineral content (BMC) were determined using dual energy x-ray absorptiometry (DXA) (GE Lunar Piximus II, Madison, WI, USA) (122). Each carcass was scanned three times and the average values were used for analysis.

2.3.2 GLUCOSE TOLERANCE AND INSULIN TOLERANCE TESTS.

To determine the effects of different sources of calcium on glucose regulation and insulin sensitivity, glucose tolerance tests (GTT) were performed at week 13 and insulin tolerance tests (ITT) at week 14 in a randomly selected subset of mice from each group. GTT was performed after overnight fasting by administration of 20% glucose (2 g/kg body weight IP); ITT was performed at noon after a 6-hour fast by IP injection of insulin (0.75 U/kg body weight) (123). For both tests, blood samples were taken from the tail and analyzed for glucose using an Ascencia Elite XL 3901G glucose analyzer (Bayer Corporation, Mishawaka, IN, USA). Glucose levels were determined at baseline, 15, 30, 60 and 120 min after injection of glucose or insulin.

2.3.3 SERUM HORMONE ANALYSES.

Insulin, leptin, adiponectin, resistin, MCP-1 and PAI-1 were measured in serum collected at week 10 of the study using mouse adipokine LINCOplex™ Multiplex Assays (Millipore, Inc., Billerica, MA, USA). Insulin-like growth factor (IGF-1) was measured by radioimmunoassay in serum collected at weeks 5 and 10 (Diagnostic Systems Laboratories; Webster, TX, USA).

2.3.4 STATISTICAL ANALYSIS.

Values are presented as mean ± standard error (S.E.). Statistical analyses were performed within each dietary fat category (i.e., HF or LF), except as noted. Repeated measures and one-way analysis of variance (ANOVA) using Tukey's Honestly Significant Difference comparison were used to assess the effects of diet on mean weekly body weight, kcal consumption and serum hormone analyses. Adiponectin values were subjected to a square root transformation before analysis to achieve a normal distribution. Body composition data were analyzed using either ANOVA or analysis of covariance (ANCOVA) with body weight as covariate. Repeated measures analysis was used to evaluate glucose and insulin tolerance tests. For all tests SPSS software was used (SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

2.4 Results

Ovariectomized mice fed the HF diets consumed more kilocalories and consistently weighed more than the mice fed the LF diets throughout the 20-week study (**Figure 2.1**). Mean kilocalorie consumption was slightly higher for mice in the HF diet; however, it did not significantly differ within the dietary fat categories ($p > 0.05$) (**Figure 2.1A**). Nevertheless, dairy

decreased the rate of weight gain, but in the HF mice only, with no effect on body weight with the LF diet (**Figure 2.1B**). Among the HF ovariectomized groups, body weights were similar until week 15 of the study, when a significant inhibition of weight gain was observed associated with dairy consumption compared to HF control. At the end of the study (week 20), mice in the HF dairy group weighed significantly less compared to both the control (p = 0.035) and supplement groups (p = 0.043). The effect of calcium on body weight was independent of the fat content in the diet, and there was no interaction between dietary fat level and calcium on body composition (**Table 2.2**). Fat mass was significantly decreased in the LF dairy group compared to the LF control (p = 0.04) and supplement (p < 0.001) groups; in addition, lean mass was significantly increased in the LF dairy group compared to the LF control (p = 0.006) and supplement (p = 0.02) groups (**Table 2.2**). Percent body fat was significantly decreased in the animals consuming dairy when compared to control for both the HF (p = 0.011) and LF (p = 0.004) diets and also decreased compared to supplement for the LF diet (p < 0.001).

Table 2.1. Diet formulations.

	Low-Fat Control	Low-Fat Supplement	Low-Fat Dairy	High-Fat Control	High-Fat Supplement	High-Fat Dairy
Diet #	D12450B	D06030301	D06030302	D12451	D06030303	D06030304
Ingredient (g)						
Casein	200.0	200.0	115.0	200.0	200.0	115.0
L-Cystine	3.0	3.0	1.7	3.0	3.0	1.7
Corn Starch	315.0	315.0	315.0	72.8	72.8	72.8
Maltodextrin	35.0	35.0	35.0	100.0	100.0	100.0
Sucrose	350.0	350.0	241.0	172.8	172.8	64.0
Soybean Oil	25.0	25.0	25.0	25.0	25.0	25.0
Lard	20.0	20.0	18.5	177.5	177.5	176.0
Milk, Nonfat, Dry			200.0			200.0
TruCal D50			50.0			50.0
Calcium Carbonate	5.5	41.5	5.5	5.5	41.5	5.5
Total[1]	1055.05	1091.05	1108.25	858.15	894.15	911.55
g (g%)						
Protein	179.0 (17.0)	179.0 (16.4)	178.8 (16.1)	179.0 (20.9)	179.0 (20.0)	178.8 (19.6)
Carbohydrate	710.0 (67.3)	710.0 (65.1)	710.0 (64.1)	355.6 (41.4)	355.6 (39.8)	355.8 (39.0)
Fat	45.0 (4.3)	45.0 (4.1)	45.1 (4.1)	202.5 (23.6)	202.5 (22.6)	202.6 (22.2)
Fiber	50.0 (4.7)	50.0 (4.6)	50.0 (4.5)	50.0 (5.8)	50.0 (5.6)	50.0 (5.5)
kcal (kcal %)						
Protein	716 (18)	716 (18)	715 (18)	716 (18)	716 (18)	715 (18)
Carbohydrate	2840 (72)	2840 (72)	2840 (72)	1422 (36)	1422 (36)	1423 (36)
Fat	405 (10)	405 (10)	406 (10)	1823 (46)	1823 (46)	1823 (46)
Total	3961 (100)	3961 (100)	3961 (100)	3961 (100)	3961 (100)	3962 (100)
kcal/g	3.8	3.6	3.6	4.6	4.4	4.3
g/kg diet						
Calcium	6.1	20.5	20.6	6.1	20.5	20.6
Phosphorous	4.6	4.6	12.3	4.6	4.6	12.3
Potassium	6.0	6.0	9.6	6.0	6.0	9.6

[1]All diets contained 50.0 g Cellulose, BW200; 10.0 g S10026 Mineral Mix; 13.0 g Dicalcium Phosphate; 16.5 g Potassium Citrate; 10.0 g V10001 Vitamin Mix; 2.0 g Choline Bitartrate; and 0.05 g FD&C dye.

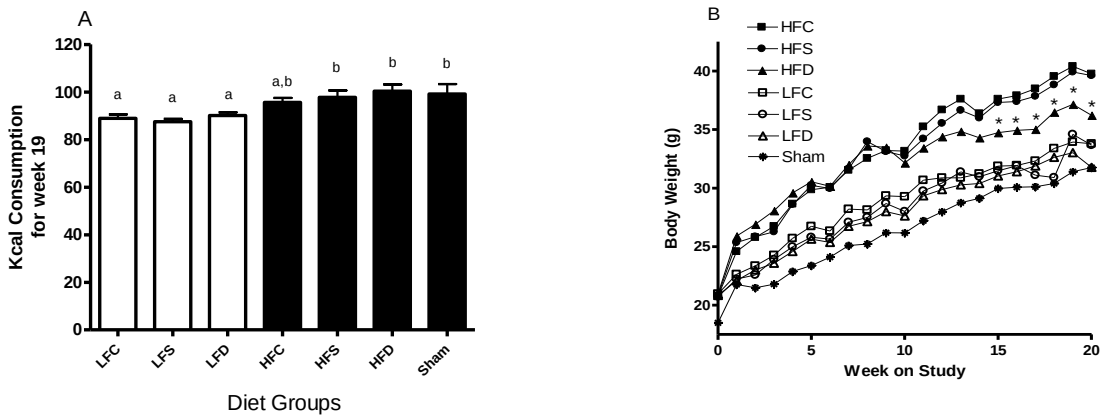

Figure 2.1. Mean kilocalorie consumption and body weights.

For the duration of the study mice consumed food and water ad libitum. Food intake (A) and body weights (B) were recorded weekly. The sham surgery (non-ovariectomized) group consumed the HF control diet. HFC, high-fat control diet; HFS, high-fat supplement diet; HFD, high-fat dairy diet; LFC, low-fat control diet; LFS, low-fat supplement diet; LFD, low-fat dairy diet. a,bBars with different superscript letters are significantly different ($p < 0.05$.) *Significantly different from the HF control diet ($p < 0.05$).

We found that supplement and dairy had different effects on bone mineral content and density, and the effects of supplement or dairy on BMC and BMD were independent of diet and diet effects on body weight (**Table 2.2**). BMC was significantly higher in both the HF and LF supplement groups compared to the respective dairy groups ($p < 0.05$). The LF supplement mice had significantly higher BMD compared to both control ($p = 0.001$) and dairy ($p = 0.001$), whereas dairy had no effect on BMD compared to the respective control ($p > 0.05$), regardless of the dietary fat category.

LF and HF groups responded differently to glucose and insulin tolerance tests, administered during weeks 13 and 14 of the study, respectively (**Figures 2.2 and 2.3**). The effect of dietary fat and/or mean body weight was stronger in these tests than that of either type of calcium. Collectively, glucose clearance was better in the LF groups compared to the HF groups. Supplement had no effect on glucose tolerance or insulin sensitivity in either the LF or HF diets. The dairy regimen improved the response to the GTT and ITT in the LF diet but not in the HF diet. Specifically, the LF dairy group exhibited the fastest glucose clearance compared to LF control and LF supplement diet categories ($p = 0.009$) by repeated measures analysis, and at the end of the test this group also displayed improved glucose tolerance ($p = 0.026$) and insulin sensitivity. In contrast to the improved glucose tolerance seen with dairy in the LF diet, mice consuming the HF dairy diet were more glucose intolerant ($p = 0.022$) and insulin resistant ($p = 0.027$) compared to the HF control.

Serum hormone levels at week 10 of the study were also differentially affected by diet (**Table 2.3**). Leptin levels in the HF control were higher than in the LF groups but were surprisingly low in the HF supplement and HF dairy groups, with no difference in serum leptin between the HF supplement and HF dairy groups. In the HF groups, serum resistin was also

decreased in supplement and dairy compared to the HF control (p = 0.033 and p = 0.012, respectively), with no difference in resistin values between supplement and dairy. No differences were found in serum leptin or resistin levels between the LF groups. Dairy increased adiponectin levels in mice on the HF diet (p = 0.006) but not the LF diet, compared to the respective control. In contrast, supplement, while not statistically different from dairy, had no effect on adiponectin levels in mice on the HF diet but surprisingly decreased adiponectin in mice on the LF diet (p = 0.022). No significant differences were found in serum insulin, MCP-1 or PAI-1 levels in ovariectomized mice in either dietary fat category (data not shown). IGF-1, a protein hormone with insulin-like effects on growth and metabolism, was measured in serum of these mice at weeks 5 and 10 of the study. By a repeated measures analysis, IGF-1 did not have a major effect among the groups and there was no significant interaction between IGF-1 and group within each dietary fat category (data not shown).

Ovariectomy had significant effects on most of the parameters measured. Although the sham surgery (non-ovariectomized) group consumed the HF control diet, the ovariectomized mice on even the LF diets generally weighed more than the sham surgery group throughout the study, and the ovariectomized mice on the HF diets weighed considerably more (**Figure 2.1**). The ovariectomized mice on the HF control diet also had greater fat mass and percent body fat and lower BMC and BMD compared with the sham surgery group (Table IV). In correlation with fat mass, serum leptin and resistin levels were higher and adiponectin levels tended to be lower in the ovariectomized mice compared with the sham surgery group on the same HF control diet (**Table 4**). The sham surgery group also displayed better glucose tolerance than any of the HF (p = 0.007) or LF groups ($p < 0.05$) (**Figure 2.2**) but responded less well to the insulin tolerance test than the LF dairy group ($p < 0.05$) (**Figure 2.3**).

Table 2.2. Effects of diets on body composition and bone characteristics in ovariectomized C57BL/6 mice

Group	Body weight[1] (g)	Fat mass[1](g)	Lean mass[1] (g)	Percent body fat[1]	Bone mineral content[2] (g)	Bone mineral density[2] (g/cm^2)
LF control	29.9 (0.8)^{a,b}	13.2 (0.8)^{a}	16.7 (0.4)^{a}	43.6 (1.7)^{a}	0.47 (0.014)^{a,b}	0.0480 (0.0005)^{a}
LF supplement	32.1 (0.9)^{b}	15.1 (1.0)^{a}	17.0 (0.4)^{a}	46.4 (2.1)^{a}	0.52 (0.024)^{b}	0.0512 (0.0007)^{b}
LF dairy	28.8 (0.9)^{a}	10.2 (0.7)^{b}	18.6 (0.5)^{b}	34.8 (1.6)^{b}	0.41 (0.012)^{a}	0.0480 (0.0005)^{a}
HF control	38.1 (1.3)^{a,b}	21.4 (1.3)^{a}	16.7 (0.5)^{a}	55.5 (2.1)^{a}	0.49 (0.022)^{a,b}	0.0474 (0.0005)^{a}
HF supplement	39.1 (1.5)^{a}	21.8 (1.4)^{a,b}	17.3 (0.6)^{a}	54.9 (2.0)^{a,b}	0.50 (0.026)^{b}	0.0494 (0.0007)^{a}
HF dairy	34.9 (1.4)^{b}	17.5 (1.2)^{b}	17.3 (0.5)^{a}	49.5 (1.8)^{b}	0.45 (0.017)^{a}	0.0474 (0.0006)^{a}
ANOVA/ANCOVA[3]						
P (Dietary Fat)	<0.0001	<0.0001	0.4217	<0.0001	0.4788	0.1012
P (Calcium)	0.0907	0.0002	0.0322	<0.0001	0.0070	<0.0001
P (Fat*Calcium)	0.5792	0.7785	0.2063	0.2598	0.1570	0.4657

[1]Means and standard errors obtained from ANOVA within dietary fat category, n=16–18. [2]Means and standard errors obtained from ANCOVA within dietary fat category with body weight as the covariate, n=16–18. [3]*P* values obtained from two-way ANOVA or ANCOVA for all diets. [a,b]Values within a dietary fat category with different superscripts are significantly different from each other, $P<0.05$

Table 2.3. Effects of diets on serum hormones in ovariectomized C57BL/6 mice

Group[1]	Leptin (ng/ml)	Resistin (ng/ml)	Adiponectin (mg/ml)
LF control	9.00 (1.1)[a]	4.1 (0.5)[a]	34.9 (5.0)[a]
LF supplement	11.3 (1.5)[a]	4.5 (0.4)[a]	21.8 (1.0)[b]
LF dairy	10.0 (1.6)[a]	4.2 (0.3)[a]	25.5 (0.8)[a,b]
HF control	18.9 (2.8)[a]	8.8 (0.8)[a]	20.9 (2.5)[a]
HF supplement	7.9 (0.6)[b]	5.9 (0.9)[b]	26.8 (2.1)[a,b]
HF dairy	7.6 (0.5)[b]	5.4 (0.6)[b]	32.3 (2.4)[b]
ANOVA[2]			
P (Dietary Fat)	0.2835	<0.0001	0.6612
P (Calcium)	0.0034	0.0251	0.3377
P (Fat*Calcium)	<0.0001	0.0083	0.0012

[1]Means and standard errors obtained from ANOVA, n=10 for all groups
[2] P values obtained from two-way ANOVA for all diets
[a,b]Values within a dietary fat category with different superscripts are significantly different from each other, P<0.05

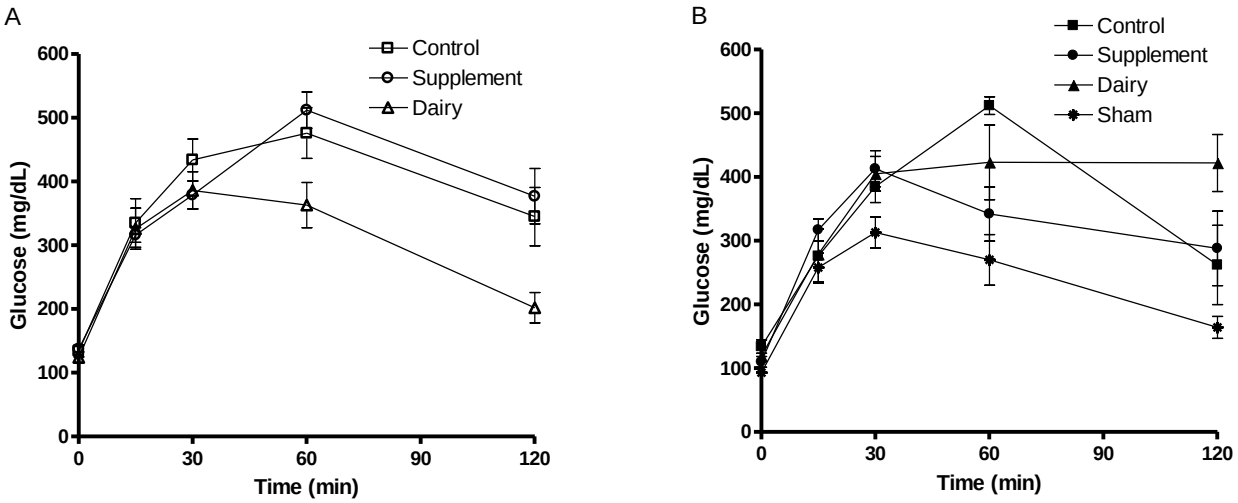

Figure 2.2. Effect of diets on glucose tolerance.

Mice were fasted overnight during week 13 on study and glucose tolerance tests (GTT) performed as described in Materials and Methods. (A) Ovariectomized mice fed LF control, supplement and dairy diets. (B) Ovariectomized mice fed HF control, supplement and dairy diets, and sham surgery (non-ovariectomized) mice fed HF control diet. Values are mean + S.E.; n = 10 mice per group.

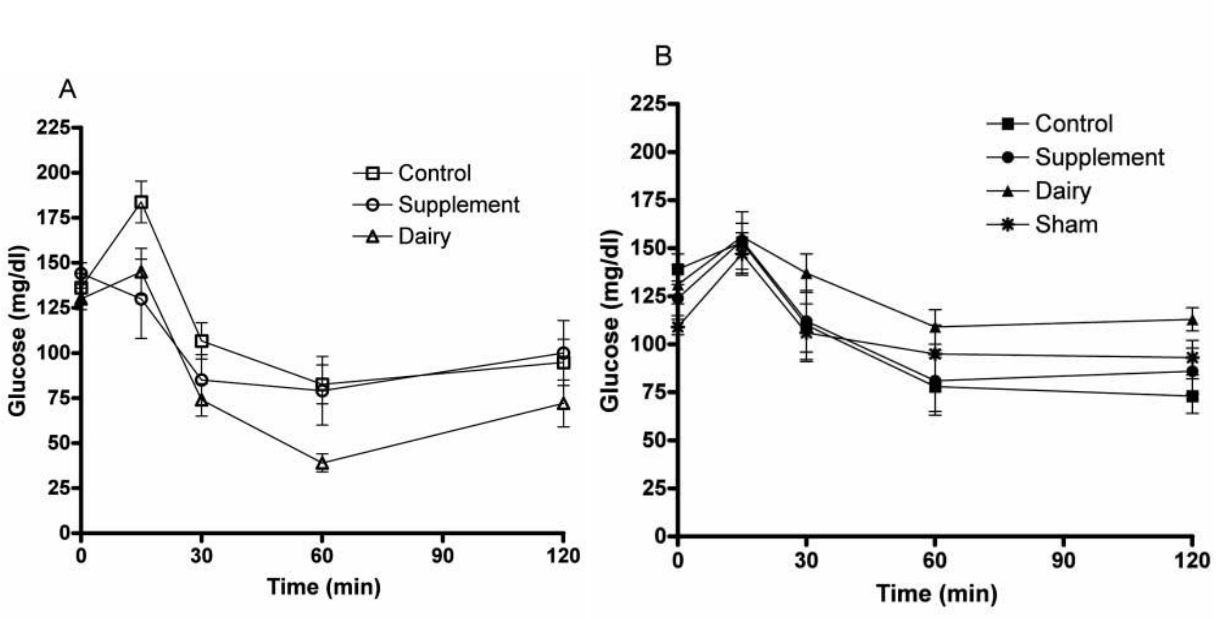

Figure 2.3. Effect of diets on insulin responsiveness.

Mice were fasted for 6 hr during week 14 on study and insulin tolerance tests (ITT) performed as described in Materials and Methods. (A) Ovariectomized mice fed LF control, supplement and dairy diets. (B) Ovariectomized mice fed HF control, supplement and dairy diets, and sham surgery (non-ovariectomized) mice fed HF control diet. Values are mean $\pm$ S.E.; n = 10 mice per group.

Table 2.4. Effects of ovariectomy on body composition and serum hormones in C57BL/6 mice fed HF control diet

Variable	Ovariectomized[2] (S.E.)	Sham[3] (S.E.)	*P*[4]
Body weight (g)	38.1 (1.3)	32.0 (2.0)	0.0097
Fat weight (g)	21.4 (1.3)	14.8 (1.8)	0.0042
Lean weight (g)	16.7 (0.5)	17.2 (0.4)	0.7544
Percent body fat	55.5 (2.1)	44.5 (3.3)	0.0067
BMC (g)	0.49 (0.022)	0.58 (0.025)	0.0055
BMD (g/cm^2)	0.0474 (0.0005)	0.0556 (0.0007)	<0.0001
Leptin (ng/ml)	18.9 (2.8)	3.0 (0.7)	0.0003
Resistin (ng/ml)	8.8 (0.8)	2.2 (0.3)	<0.0001
Adiponectin (µg/ml)	20.9 (2.5)	33.8 (6.9)	0.0952

[1]Data for ovariectomized mice from Tables 2 and 3
[2]n=17 for body composition variables; n=10 for serum hormones
[3]n =10
[4]P value from t-test

2.5 Discussion

This study compared the effects of supplemental calcium and dairy calcium on body weight and composition, glucose and insulin metabolism, and serum adipokines in a mouse model of postmenopausal obesity. The results support the hypothesis that consumption of dairy influences body composition by decreasing the rate of weight gain. In the present study, kilocalorie consumption did not differ within the respective dietary fat categories, and yet with the HF diet differences in body weight and body composition were evident in the mice consuming the dairy diet. As has been shown in some previous studies (124), decreased body weight in the HF dairy group was associated with decreases in fat mass and percent body fat rather than lean tissue. In the LF dairy group fat mass and percent body fat were also decreased compared to the LF control, but the decreased body fat mass was partially offset by increased lean mass, resulting in no significant effect on body weight.

Mechanisms by which dietary calcium may affect body weight are not understood. Among the mechanisms suggested for an effect of dietary calcium on body weight is a reduced Ca^{2+} influx into the adipocyte, mediated by calcium suppression of 1,25-dihydroxy vitamin D production and resulting in an increase in lipolysis and thermogenesis and hence weight loss (125). Alternatively, calcium is known to bind to lipids, and this binding contributes to increased fecal fat and decreased fat absorption (126). In the present study, however, while dairy significantly affected body composition, calcium supplement was ineffective and had no effect decreasing body weight, fat mass, lean mass or percent body fat in either the HF or LF diets. Therefore, the beneficial effects of dairy on body weight may have been due to components other than calcium, since similar effects were not observed in the supplement groups consuming the same amount of calcium but as calcium carbonate. Some studies suggest that whey peptides,

calcitropic hormones, and/or sphingolipids in milk may be responsible for the body weight-lowering effect of dairy in humans (124). Calcium in milk is usually associated with protein and, therefore, the calcium content is dependent on the protein content of the milk (117). Moreover, milk composition from cows is not constant, and proteins, fat, lactose, vitamins and minerals in milk may vary depending on many factors. In the present study, the study diets were formulated to ensure that they contained the same amounts of protein and calcium, allowing for comparison of dietary calcium sources under both HF and LF conditions. However, the source of the protein in the dairy diets differed from that in the control and calcium supplement diets, in that non-fat dried milk was substituted for a portion of the casein component. Therefore, the dairy diets had a different amino acid composition because of the contribution of the whey proteins in milk. Compared to other dietary proteins, whey contains the highest concentrations of the essential branched-chain amino acids (BCAAs), especially leucine (127). Unlike other amino acids, BCAAs are not metabolized in the liver, and dietary intake directly affects circulating levels and availability in peripheral tissues. Furthermore, leucine stimulates protein synthesis at the level of translational initiation, both through the mammalian target of rapamycin (mTOR) cellular signaling pathway and through an mTOR-independent kinase (128). Given the pivotal role of mTOR signaling in obesity and its impact on a number of chronic diseases (129), further study of the possible contributions of amino acid composition to the beneficial effects of dairy consumption on body composition is warranted.

Calcium and bone metabolism in mammals is regulated by the vitamin D receptor–1,25-dihydroxyvitamin D complex (130), and dietary calcium, especially dairy calcium, is known to regulate the level of 1,25-dihydroxyvitamin D, the active form of Vitamin D (18). In rodents this control varies with strain (131), and in C57BL/6 mice calcium metabolism is highly dependent

on vitamin D status (132). This study focused on dietary calcium sources without altering basal vitamin D status. Because vitamin D levels or calcium absorption rates could not be assessed in these animals, it is not known whether calcium metabolism differed between dairy and supplement. However, this study showed that the presence of either dairy or supplemental calcium did not affect food intake and that, in the case of dairy, the beneficial effects on body composition may have been due to components other than calcium. Future studies should address this issue and identify possible mechanisms responsible for the differential effects of dairy and supplement calcium observed in this study.

Several studies attribute optimal bone health to consumption of milk and dairy products (110, 133). The U.S. Department of Health and Human Services 2005 *Dietary Guidelines for Americans* recommends 3 servings of milk products per day to increase BMD. Recent evidence supports the idea that dietary calcium, primarily from dairy, plays a major role in preventing osteoporosis and loss of BMD in healthy adults (133, 134). However, there is much less evidence and a lack of agreement in the literature regarding the role of dairy after menopause, when calcium absorption is decreased independently of vitamin D status (135). These results suggest that dairy calcium may not be the first choice when targeting bone health after menopause. In this model of postmenopausal obesity dairy did not improve BMD, in contrast to supplement calcium, which improved BMD in the LF diet. This supports findings from other studies concluding that the emphasis on dairy to improve bone health may be overstated (120). However, BMD was significantly lower in all diet groups compared with the sham (non-ovariectomized) mice, signifying once more the protective role of ovarian hormones in the maintenance of bone density.

The effect of consumption of dairy products on insulin and metabolism continues to be a center of debate (136, 137). Data from the Epidemiological Study on the Insulin Resistance Syndrome (DESIR) showed a beneficial effect of dietary calcium on insulin levels in women (111). Inverse associations between dairy consumption and type 2 diabetes have been reported previously and have been attributed to low-fat dairy products only (138). In the present study it was observed that dairy was effective in improving glucose tolerance and insulin sensitivity, but only with the LF diet, while calcium supplement had no effect. The beneficial effects of dairy on glucose metabolism may have been due to the different amino acid composition contributed by the whey proteins, as BCAAs, and leucine in particular, have been linked to maintenance of glucose homeostasis (127). In contrast, the HF dairy diet increased glucose intolerance and insulin insensitivity. These results suggest that either fat in the diet or increased adiposity hinders the dairy modulation of glucose and insulin metabolism in mice. Future studies isolating this effect will be needed to expand the characterization of the influence of dairy consumption on metabolism.

This study showed a clear interaction between dietary calcium and dietary fat/increased adiposity on several endocrine markers associated with insulin resistance and body weight. Leptin and resistin are two adipose-secreted proteins known to play a role in metabolism, and their serum levels increase with increased adiposity. In addition, resistin may contribute to insulin resistance, and some reports indicate that resistin may be a determinant factor in the progression to type 2 diabetes mellitus in obesity (104). Resistin may also be a link between inflammation and insulin resistance, as has been shown in rodents (139), although the human data are not conclusive. Adiponectin is another of the key adipokines related to body weight and metabolism; however, serum levels of adiponectin decrease with increased body weight, and

leptin and adiponectin appear to exert opposite effects in metabolism. Recent studies have focused on the leptin-adiponectin ratio as a potential marker for metabolic diseases, including type 2 diabetes (140). In the present study, dietary calcium, regardless of source, decreased leptin and resistin and increased adiponectin, but only in the HF diet. The effect of calcium supplement on adiponectin levels in the HF diet was not significant, while in the LF diet calcium supplement actually decreased adiponectin in this study. However, the differences in adipokine levels with different sources of calcium did not correlate with improvements in glucose tolerance or insulin sensitivity. These results suggest that, while dietary calcium may help counteract some of the deleterious effects of obesity on these endocrine markers, the beneficial effect of dairy on glucose metabolism observed in the LF mice may not be related to circulating levels of these hormones. Given the dramatic increase in obesity rates in recent decades, the prevalence of postmenopausal obesity can be expected to increase as the current cohort of women enters menopause. Postmenopausal obesity is a well-known risk factor for several chronic diseases, including type 2 diabetes, metabolic syndrome, and some cancers. This study in an animal model of postmenopausal obesity lends support to the relevance of dairy consumption and dietary calcium in the maintenance of body weight and composition, but more work is needed to clarify their roles.

Chapter 3: The Enhancing Effects of Obesity on Mammary Tumor Growth and Akt/mTOR Pathway Activation Persist After Recent Weight Loss and are Reversed by RAD001

3.1 Abstract

Obesity is an established epidemiologic risk factor for postmenopausal breast cancer and is also associated with enhanced breast cancer progression and activation of Akt/mTOR signaling. However, it is not known if restoration of normal weight in obese, postmenopausal individuals can reverse the obesity-associated mammary tumor progression and/or Akt/mTOR pathway activation. To evaluate this, 70 ovariectomized (OVX) female C57BL/6 mice were fed a control diet, a calorie restricted (CR) regimen, or a diet-induced obesity (DIO) regimen for 17 weeks, at which time the DIO mice were switched to the control diet. MMTV-Wnt-1 mammary tumor cells ($5x\ 10^4$ cells/mouse) were orthopically injected all mice at week 20 in the mammary fat pads. Two weeks later, all mice began a 4-week regimen of either RAD001 (10 or 15 mg/kg) or placebo by oral gavage. Relative to mice receiving control diet, the CR mice were leaner and had decreased serum levels of leptin and IGF-1, decreased tumor growth, and decreased phosphorylation of Akt/mTOR pathway components including S6. In contrast, tumor growth and Akt/mTOR signaling were enhanced in formerly obese mice, relative to control mice, despite restoration of weight, adiposity and serum hormone levels. RAD001 (10 mg/kg BW) effectively decreased tumor growth in the CR and control group (by 81% and 61% respectively), and to a lesser extent in the formerly obese (48%). However, a higher dose of RAD001 (15 mg/kg BW) more effectively decreased tumor growth in the formerly obese group (76%). In addition, serum

collected from these mice was used in vitro and demonstrated that adipocyte-secreted factors contribute to tumor invasiveness in spite of weight loss. These results suggest restoration of normal weight in obese individuals does not reverse the enhancement of tumor progression or the activation of Akt/mTOR signaling, at least in our transplanted Wnt-1 mammary tumor model and in the timeframe studied. However, treatment with the mTOR inhibitor RAD001 did reverse the effects of obesity on mammary tumor development and mTOR pathway activation, suggesting a combination of lifestyle and pharmacologic strategies may be effective for breaking the obesity-breast cancer link.

3.2 Introduction

Findings from both epidemiological and preclinical studies suggest that obesity is a risk factor for postmenopausal breast cancer and is also associated with poor breast cancer prognosis, shorter disease-free and overall survival (23, 141). Although weight loss can reduce obesity-associated risk factors for a variety of diseases, such as diabetes and cardiovascular disease (142, 143), few studies have been done to provide direct evidence of benefits from weight loss in obese indiciduals (144). Most studies on the beneficial effects of weight loss have focused on changes in levels of circulating hormones, while considerably less is known about how changes in energy balance-related hormones/growth factors affect signaling pathways involved in cancer. However, there are no reports of the effect of weight loss on breast cancer progression. A previous animal study also showed that as obesity increases, in response to a high-fat diet, adipocyte gene expression pattern is altered and a number of genes do not return to the pre-obese state following weight reduction (145).

Calorie restriction (CR) is the most potent dietary regimen to prevent and/or reverse obesity and inhibit tumor growth (146). In animal models, CR regimen is based on a low-calorie diet that supplies all of the nutrients to sustain life (146, 147). The beneficial effects of CR on tumor growth are attributed to low body fat that results in decreased secretion of mitogenic hormones and cytokines such as, estrogen, leptin, resistin, insulin, and MCP-1 (141), while increased levels of adiponectin as result of CR, has been shown to inhibit activation of signaling pathways implicated in cell grow and proliferation (148).

Body weight reduction is often accompanied by alterations in growth factors and mitogenic hormones such as insulin, estrogen, and insulin like growth factor-1 (IGF-1) that are

elevated in the obese state. These hormones/growth factors activate signaling pathways that converge at mTOR, a serine/threonine kinase downstream of Akt that plays a critical role in cell survival and proliferation. Akt/mTOR activation leads to the phosphorylation of its downstream targets including ribosomal p70S6 serine/threonine kinase (S6K1) and eukaryotic initiation factor (EIF)-4E binding protein (4E-BP1), resulting in an increase in mRNA translation and cap-dependent protein synthesis, respectively. Previous studies from our lab have shown that lean mice exhibiting lower levels of these obesity related hormones display decreased steady state signaling through AKT/mTOR compared to obese mice (149). Furthermore, decreased activation of AKT/mTOR is associated with reduced mammary tumor growth (67).

In the present study, we have used ovariectomized C57BL/6 mice to model the postmenopausal state and injected them with syngeneic tumor cells obtained from the MMTV-Wnt-1 transgenic mouse. Similar to postmenopausal women, ovariectomized mice experience increased weight gain and decreased bone mineral density (141), while, like many breast cancers, MMTV-Wnt-1 mouse tumors express estrogen receptors (150). Therefore, the aim of this study was to determine the effect of weight loss with and without pharmacologic inhibition of mTOR signaling, on mammary tumor growth and mammary epithelial cell signaling, in diet-induced obese ovariectomized mice. Results from this study indicate that after rapid weight loss, there is a persistent effect of obesity on tumor growth characterized by continued activation of Akt/mTOR. In addition, the mTOR inhibitor RAD001 is effective at inhibiting persistent effects of obesity following weight loss on postmenopausal breast cancer.

3.3 Materials and Methods

3.3.1 Mice and Diets

All animal protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and carried out in compliance with all guidelines and regulations. Six-week-old ovariectomized female C57BL/6 mice (n=70) were obtained from Charles River Laboratories, Inc. (Animal Production Area, NCI-Frederick, Frederick, MD), and placed on a chow diet. Ovariectomized mice were used to mimic the postmenopausal state, which is characterized by decreased levels of circulating estrogen, loss of bone mineral density, and cessation of the estrous cycle (151). All mice were individually housed on a 12-hour light/dark cycle, and consumed food and water ad libitum. Food intake was measured twice a week, and body weights were measured weekly. Following a one week acclimation period after arrival, mice were randomized to the following diet regimens: 1) control (n=20; 10 kcal % fat D12450B; Research Diets, New Brunswick, NJ); 2) calorie restriction (CR) (n=20; D0302702); and 3) a diet-induced obesity (DIO; n=30; D12492; Research Diets, Inc, New Brunswick, NJ). The CR group consumed a modified diet administered in daily aliquots providing 30% fewer calories from carbohydrates compared to the control diet, with all other components formulated on an isonutrient basis when intake was limited to 70% of mean kcal consumption of the diet control group. At week 17, DIO mice were switched to the control diet. Control and lean mice continued in their diets for the duration of the study3.3.2 Body composition analyzes
Body composition was measured on all mice at weeks 17 and 20 of diet treatments by quantitative magnetic resonance (qMR) (Echo Medical Systems, Houston TX). Endpoints included lean mass, fat mass, body weight, and percent body fat.

3.3.2 Body Composition Analyses

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3.3.3 MMTV-Wnt-1 Tumor Cell Injection and RAD001 Administration

At week 20, all mice were injected with 5x10^4 syngeneic MMTV-Wnt-1 mammary tumor cells as previously described (152). Briefly, spontaneous mammary tumors from MMTV-Wnt-1 transgenic mice on a C57BL/6 background were mechanically dispersed to form a cell suspension and viable cells counted and orthotopically injected into the fourth mammary fat pad. Two weeks after tumor injection, 10 mice/diet group received RAD001 (10 mg/kg body weight in a volume of 1ml/100g of BW, dissolved in water) and 10/diet group received a placebo solution also dissolved in water by oral gavage 2x/week for 6 weeks. The remaining 10 DIO mice received a higher dose of RAD001 (15 mg/kg body weight). Tumor growth was measured 2x/week with skinfold calipers and tumor volume was approximated using the formula $4/3\pi r1^2r2^2$ (153)At study endpoint (week 28), the mice were anesthetized with isofluorane for terminal blood collection via cardiac puncture. Whole blood was allowed to clot at room temperature for 30 min prior to centrifugation at 1000 x g for 10 min. The serum was removed, aliquoted in 25 ul quantities and stored at -80° C for analyses. Tumors were excised, weighed, and randomly assigned to be either formalin fixed and paraffin-embedded or to be flash frozen in liquid nitrogen and stored at -80 °C until further analysis

3.3.4 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemistry (IHC) was performed on a random sample of formalin-fixed tumors (5/group) collected as described (71). Slides were incubated with primary antibodies for the following proteins: total and phospho Akt, phospho-ribosomal S6 and mTOR, phospho-P70S6K, pHH3, and CD31, (Cell Signaling Technology, Danvers, MA), according to manufacturer's protocol.

In all of the samples, proliferating areas were analyzed with the exclusion of necrotic ones. For phospho-S6, phospho-Akt, phospho-mTOR, and CD31, the total number of mitoses in mammary tumors per three- 20X-magnification fields was counted in a total area of 0.1 mm^2 for each field. For total mTOR, phospho-P70S6K, and pHH3, slides were scanned into the Aperio System (Vista, CA) using the standard ImageScope algorithms for total pixel count (total mTOR) and for nuclear immunostaining (phospho- P70S6K, pHH3). For all other immunostainings, % positive nuclei/cells protein expression was evaluated according to staining level and distribution.

3.3.5 SERUM HORMONE MEASUREMENT

Leptin was measured in serum using mouse adipokine LINCOplex®Multiplex Assays (Millipore, Inc., Billerica, MA) and analyzed on a BioRad Bioplex 200 analysis system (Biorad, Inc. Hercules, CA). Serum insulin-like growth factor-1 (IGF-1) concentration was measured by radioimmunoassay in 25 μl of serum (Diagnostic Systems Laboratories, Webster, TX, USA) as previously described (141).

3.3.6 CELL CULTURE STUDIES

Cells and reagents

The MMTV-Wnt-1-G4 cell line (hereafter called WG4) was previously cloned from a spontaneous mammary tumor from an MMTV-Wnt-1transgenic mouse (152). Cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and penicillin-streptomycin (100 U/ml and 0.1 mg/ml respectively; Sigma-Aldrich) and kept in a humidified atmosphere of 5% CO_2 at 37ºC.

In vitro proliferation assay

Cell proliferation was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) dye conversion at 570 nM according to manufacturer's protocol. Briefly, WG4 cells were seeded at a density of 3 $x10^3$ cells/well on 96-well flat bottom plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to adhere for 24 h. Cells were cultured in 10% FBS, serum free, or 1% mouse serum collected from the lean, control, and formerly obese mice described above. After 48 h of continuous culture, 50 µl of MTT (5 mg/ml in PBS) was added to each well. After 2 h incubation at 37ºC, cells were lysed by the addition of DMSO.

Migration assay

WG4 cells were grown in 75-cm^3 tissue culture flasks (Falcon Labware, BD; Franklin Lakes, NJ) to 80% confluence and harvested with 0.25% Trypsin (Sigma–Aldrich; St. Louis, MO). A chemoattractant (1% serum from lean, control, and formerly obese mice) was added to RPMI media in a 24-well invasion chamber. Cells were seeded (5x10^4 cells/ml) on a Boyden chamber in serum free RPMI and inserted to the wells. After 30-h incubation at 37ºC in 5% CO2,

the non-invading cells were removed from the upper chamber with a cotton swab. Cells that migrated through the matrix of the upper chamber were stained and fixed with 1% crystal violet for 30 min at room temperature. Five fields of adherent cells were randomly counted in each well with a Nikon Diaphot-TMD inverted microscope at 20X magnification.

3.3.7 Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) using Tukey's Honestly Significant Difference comparison was used to assess the effects of diet and RAD001 treatment on mean tumor size and serum hormone levels. For the proliferation and migration assays, means were compared across treatment groups using Student's t test. Formal statistical tests were conducted using SPSS software (SPSS Inc., Chicago, IL), and $P \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Effect of diet treatments on body weight, body composition and hormone levels

As expected, the diet-induced obesity regimen led to an increase in adiposity. After 17 weeks on the diets, DIO mice were significantly heavier (47.4 g ± 1.1) than control mice (33.7 g ± 0.6). In contrast, the CR mice (25.3 g ± 0.4) had significantly lower body weights than the control mice (**Figure 3.1A**). DIO mice displayed significantly more fat mass and had a higher percent body fat (54.4%) compared to control mice (41.7%) ($p<0.001$). In the CR mice both of these parameters were significantly decreased ($p<0.05$) compared to control mice. Once the DIO mice were switched to the control diet (end of week 17), they rapidly lost weight (average loss was 12.1 g, an 18% reduction) and were virtually identical to the control mice by week 20 (**Figure 3.1B**). Other body composition parameters also returned to control levels in these formerly obese mice. Body weight and body composition parameters did not change during this same period (weeks 17-20) in either the CR or control mice, nor were these parameters affected by treatment with RAD001.

Elevated levels of both leptin and IGF-1 can activate Akt/mTOR signaling in central and peripheral tissues including adipocytes (29). Therefore we performed serum analyses to determine if these obesity-related hormones were associated with mammary tumor growth in the formerly obese mice. As expected, serum levels of leptin and IGF-1 were decreased in CR when compared to control mice. However, no differences were observed between formerly obese and control mice. RAD001 (10 mg/kg) did not affect leptin levels in CR or controls; in contrast, formerly obese mice showed increased leptin levels in response to RAD001 treatment. In

addition, RAD001 (10mg/kg) increased IGF-1 levels in all dietary groups (**Figure 3.2C**). This effect previously has been reported as an autocrine loop in response to mTOR inhibitors (154).

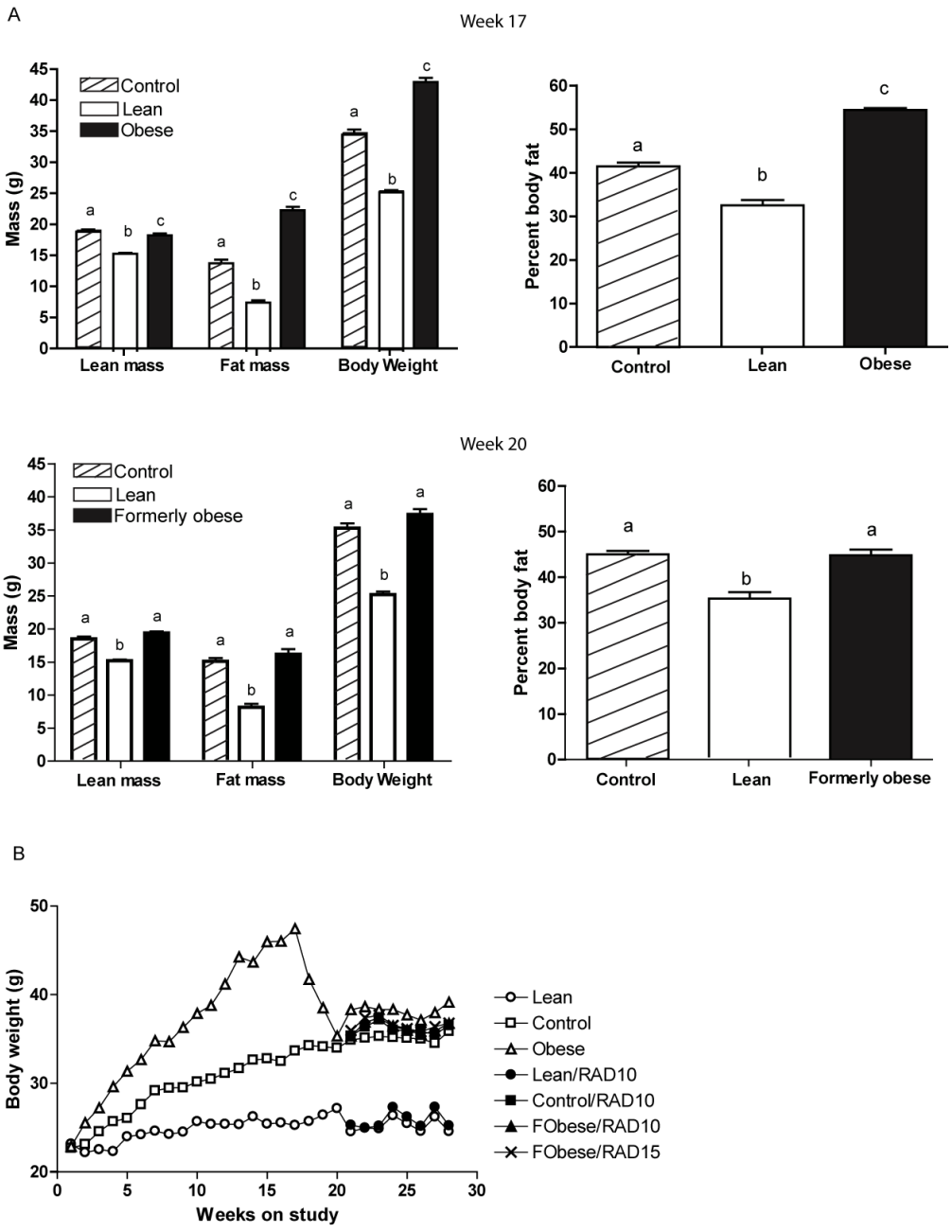


Figure 3.1. Body weight and body composition of C57BL/6 OVX mice.

Ovariectomized C57BL/6 mice in three diet groups (lean, control, DIO). (A) At week 17 and 20 on the diet regimens, body composition was assessed by quantitative magnetic resonance. (B) Body weight is not affected by RAD001 treatment. Values represent mean ± SEM from groups (n=20-30/group). Different letters indicate significant differences (p<0.05).

3.4.2 INCREASED MMTV-WNT-1 MAMMARY TUMOR GROWTH IN FORMERLY OBESE MICE.

Studies from our group have previously reported the beneficial effects of CR on transplanted Wnt-1 tumor growth (141, 149). In the present study, CR mice had significantly smaller tumors relative to the control and formerly obese mice, confirming the protective effect of CR (**Figure 3.2A**). However, tumor growth was significantly enhanced in formerly obese mice ($p<0.05$) compared to the control and CR groups. This is particularly surprising considering that the body weight in formerly obese mice was comparable to the control group at the time of tumor cell injection. At the end of the study, an ex vivo measurement of tumor weight and size corroborated increased tumor size and weight in formerly obese mice (**Figure 3.2B**), while the lean mice had smaller tumors compared to the control mice.

RAD001 (10 mg/kg) effects in CR mice were enhanced and tumor weight was decreased by ~82%. There was a 60% decrease in tumor weight in the control mice and 48% decrease in the formerly obese mice. However, this partial resistance was overcome with the administration of a higher dose of RAD001 (15 mg/kg) and tumor weight reduction was ~76% in the formerly obese mice that received 15 mg/kg.

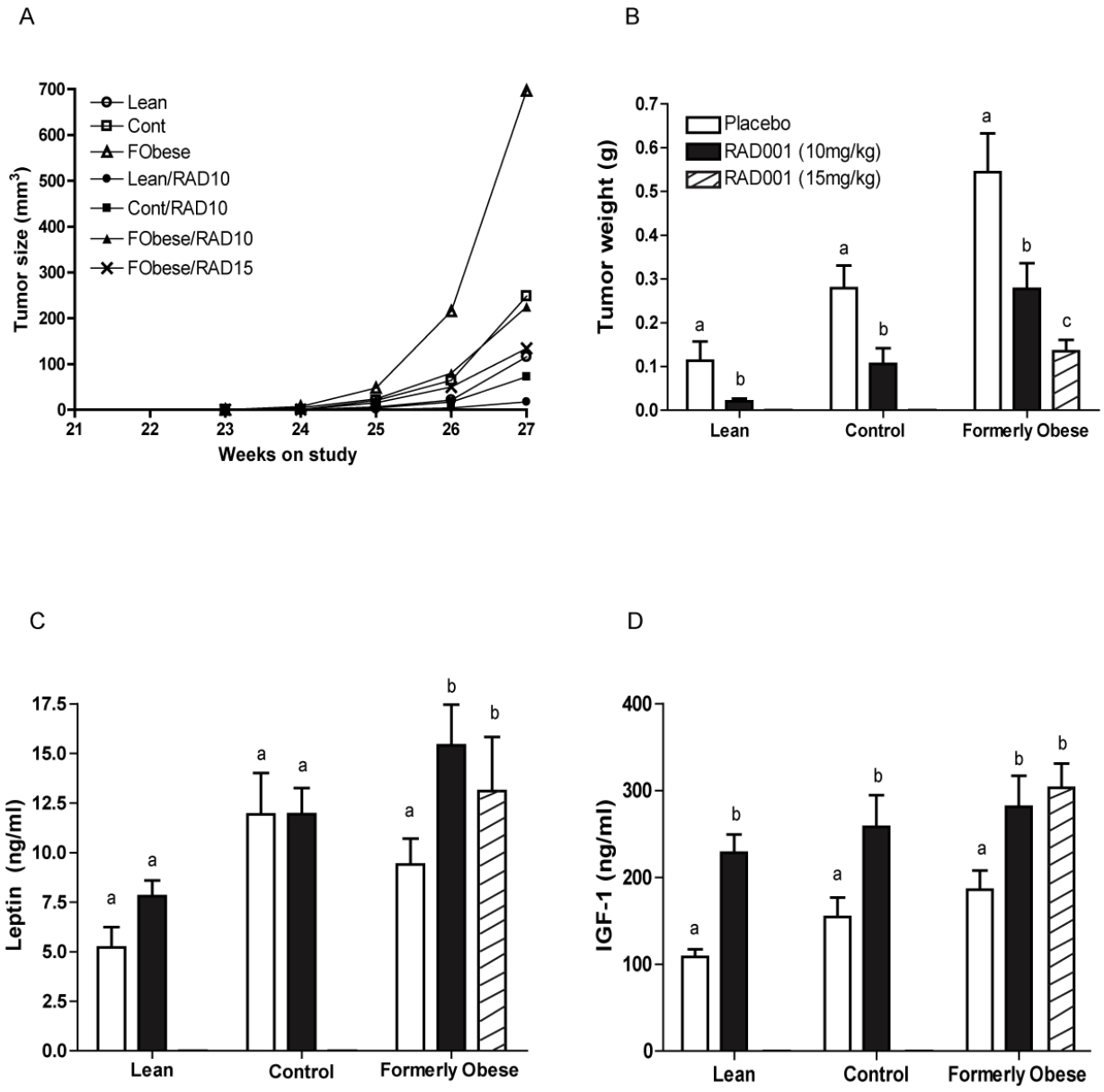


Figure 3.2. Wnt-1 mammary tumor growth in C57BL/6 ovariectomized mice.

Wnt-1 mammary tumor growth in lean, control, and formerly obese mice receiving placebo or RAD001 (10mg/kg) gavage treatment (A). A second group of formerly obese mice received a higher dose of RAD001 (15 mg/kg). (B) Tumor weight at the end of the study shows the effect of RAD001 on mammary tumors from these mice. Serum leptin (C) and IGF-1 (D) at endpoint. Values represent mean ± SEM from groups (n=10/group). Different letters indicate significant differences ($p<0.05$).

3.4.3 MMTV-WNT-1 ADENOCARCINOMAS ARE LESS DIFFERENTIATED AND MORE VASCULARIZED IN FORMERLY OBESE MICE

The histopathologic analysis of these tumors showed that regardless of the dietary group, all tumors induced by syngeneic injections of MMTV-Wnt-1 mammary tumor cells were moderately to well differentiated mammary adenocarcinomas with a predominant tubular pattern but also presenting areas with papillary or cystic differentiation. However, the degree of differentiation and the nature of the tumor stroma and vascularization showed clear differences among the groups. For example, the tumors in the CR group were the more differentiated and the tumoral masses were formed by nests of neoplastic cells separated by the normal stroma of the host (mainly loose connective tissue containing adipose tissue). Only 2/5 showed some coalescence among the different cell nests.

These morphological characteristics are consistent with a slow growing tumor in which the individual monoclonal or oligoclonal tumor nests produced by the injection of MMTV-Wnt-1 cells remain as isolated tumors masses surrounded by a poorly vascularized host stroma. In contrast, the cells injected in the control and formerly obese mice have grown to form solid masses separated by septi of well vascularized dense connective stroma. This was particular evident in the formerly obese mice. All tumors presented apoptotic figures although they were more prominent in the lean mice. On the other hand in the control mice areas of central necrosis was observed in the larger tumoral masses indicating that the tumor growth exceeded the capacity of vascularization. Thus, the different diets not only affected the rate of proliferation of the tumor but seemed to affect the degree of differentiation and the capacity of the tumor to induce a protumorigenic stroma. These histopathological observations were fully consistent with the differences between the growth curves shown in Figure 3.1.

The immunohistochemical mitosis marker phospho-Histone H3 (pHH3) and the vascular endothelial CD31 marker were also evaluated by IHC across the different dietary groups.

No differences in proliferation among the dietary groups were apparent with Ki-67 staining (Suppl. Fig. 1). However, with the marker phospho-Histone H3 (pHH3), staining was greater in tumors from control and formerly obese mice than from CR mice within the placebo (untreated with RAD001) group. In the placebo groups, the cells that stained positive for pHH3 were generally scattered throughout the whole tumor in lean mice compared with a clustered pattern in tumors from control and formerly obese mice (**Figure 3.3**). There was a good correlation between the mitotic rate assessed on hematoxylin and eosin and the mitotic rate using pHH3 expression. Staining for pHH3 was lower in lean (average 50, range 30-82) and control (average 56, range 37-79) when compared to formerly obese (average 149, range 75-343).

As discussed above, H&E staining of these tumors revealed the presence of few and immature blood vessels in the lean mice, in contrast to the control and formerly obese mice that showed more mature and larger blood vessels. These observations were confirmed using the vascular endothelial marker CD31 (**Figure 3.3**).

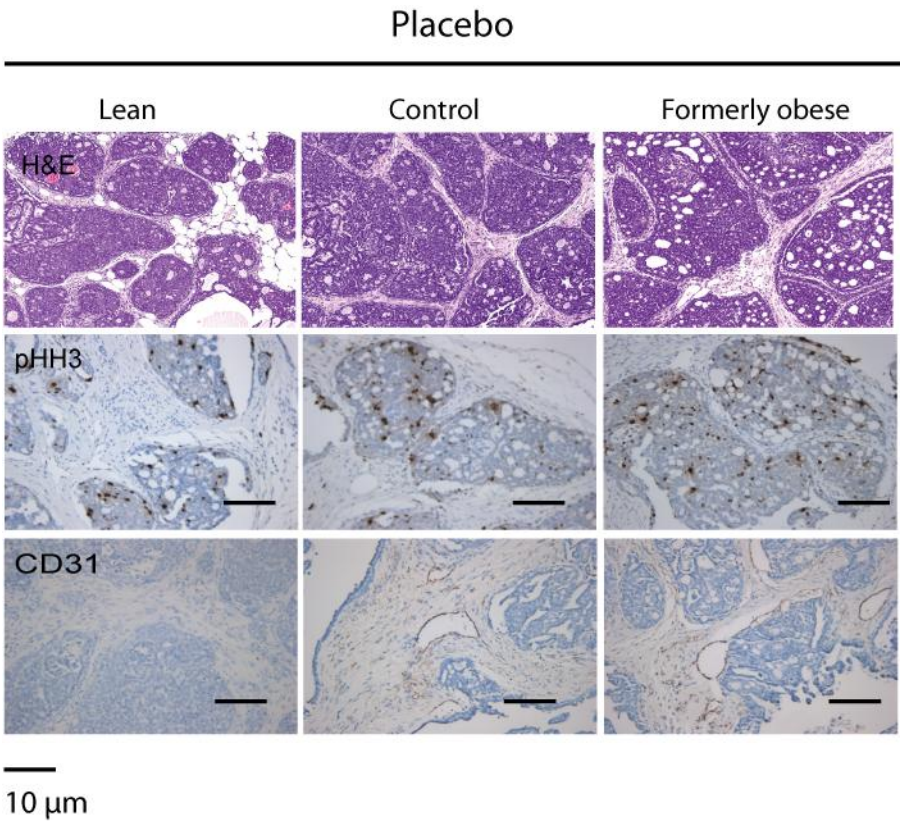


Figure 3.3. Wnt-1 mammary tumors from mice receiving placebo.

H&E staining of Wnt-1 mammary tumors show that former obesity contributes to a more heterogeneous tumor. Positive nuclear staining for pHH3 is higher in control and formerly obese mice than in lean mice. No positive membrane CD31 staining is observed in lean mice, while control and formerly obese display positive staining for CD31.

3.4.4 MAINTENANCE OF AKT/MTOR ACTIVATION PROMOTES MMTV-WNT-1 MAMMARY TUMOR GROWTH IN FORMERLY OBESE MICE

To determine diet-dependent differences in activation of components of the Akt/mTOR signaling pathway, mammary tumors from the placebo groups were analyzed by IHC for protein phosphorylation and protein expression changes in Akt, mTOR, S6 ribosomal protein, and p70S6K.

As expected based on previous studies, IHC analyses of mammary tumor sections from placebo groups revealed decreased phosphorylated protein expression (without changes in total) of Akt, mTOR, S6 ribosomal protein, and p70S6K in the CR group compared to the control group (**Figure 3.4**). However, IHC staining revealed that mammary tumors from formerly obese mice (relative to control tumors) showed persistent Akt/mTOR activation. As shown in figure 3.4, IHC analyses of mammary tumor sections revealed increased phosphorylated protein expression (without changes in total) of Akt, mTOR, S6, and P70S6K in the formerly obese group compared to the control group, even though the formerly obese mice lost 18% of their weight and weighed the same as the control mice.

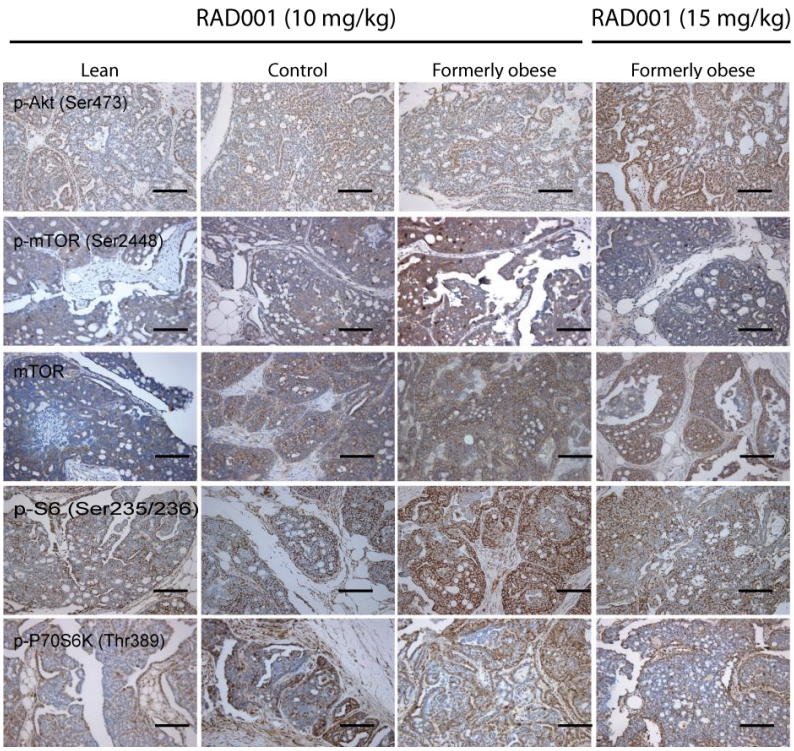


Figure 3.4. Immunohistochemistry of Wnt-1 mammary tumors in mice receiving RAD001.

H&E staining shows that RAD001 (10mg/kg) did not affect the pattern of differentiation in tumors from lean, control, and formerly obese mice, while mice treated RAD001 (15mg/kg) showed a more necrotic tumor. Immunohistochemistry of components of the Akt/mTOR signaling pathway showed nuclear and cytoplasmic staining that was almost no different between lean, control and formerly obese mice for phospho-akt(Ser 473). This figure shows decreased staining of phospho-mTOR (Ser2448), phospho -S6 (Ser235/236), and phospho –P70S6 (Thr389) in tumors from lean mice, while formerly obese mice show increased staining while compared to controls.

3.4.5 Increased MMTV-Wnt-1 mammary tumor growth in formerly obese mice is reversed by RAD001 treatment

Our results showed that phosphorylation of Akt/mTOR signaling components remains activated following weight loss in formerly obese mice. We further investigated whether mTOR inhibition by RAD001 would offset this effect. RAD001 treated tumors from CR, control, and formerly obese mice were analyzed by IHC for protein phosphorylation and protein expression changes in Akt, mTOR, S6 ribosomal protein, and p70S6K. IHC analyses were performed on several components of the Akt/mTOR signaling pathway. RAD001 decreased Akt (Ser473) phosphorylation across the diet groups, but no apparent differences were observed between control and formerly obese tumors. Occasionally the staining was more intense in cells surrounding vessels. The same pattern of staining was also observed in the downstream effectors of mTOR, p-S6 (Ser236/236) and p-P70S6K (Thr389), indicating that mTOR signaling is activated in formerly obese mice (**Figure 3.5**) and that RAD001 was able to offset this effect. However, p-P70S6K was dramatically decreased in all groups and a further decrease in staining was observed in tumors from formerly obese mice receiving the high dose of RAD001.

In the case of phospho-mTOR, a more moderate decreased staining was observed in mammary tumors from lean, control, and formerly obese mice.

IHC analysis of these tumors showed that RAD001 did not affect the pattern of differentiation but increased the frequency and size of cystic areas, particularly in the lean and control mice as well as in the formerly obese with the higher dose of RAD001. RAD001 significantly reduced tumor size and weight in all diet groups. Furthermore, tumors from formerly obese mice that received the high dose of RAD001, showed a change in pattern in which smaller lobules are

separated by thick septi of connective tissue, including in some cases adipose tissue. This indicates that RAD001 (15 mg/kg) contributed to slower tumor cell growth.

In light of the dramatic increase in tumor growth seen in formerly obese mice, we further investigated by IHC whether RAD001 will decrease pHH3 protein expression and vessel formation. In the RAD001-treated groups, pHH3 staining in CR tumors did not change (average 24, range 21-26 per 3 fields). However, there was a dramatic reduction in control tumors (average 20, range 15-26 per 3 fields). pHH3 staining in formerly obese tumors was not decreased compared to controls (average 40, range 27-50 per 3 fields). Nevertheless, tumors from formerly obese mice receiving the high dose of RAD001 had a dramatic reduction in pHH3 staining (average 23, range14-29 per 3 fields). RAD001 (10 mg/kg) was also effective at decreasing vessel formation in both CR and control tumors. Moreover, CD31 staining was not decreased in formerly obese mice tumors treated with RAD001 (10 mg/kg), although the higher dose of RAD001 (15 mg/kg) decreased CD31 staining in formerly obese mice to the level of control tumors treated with the regular dose of RAD001.

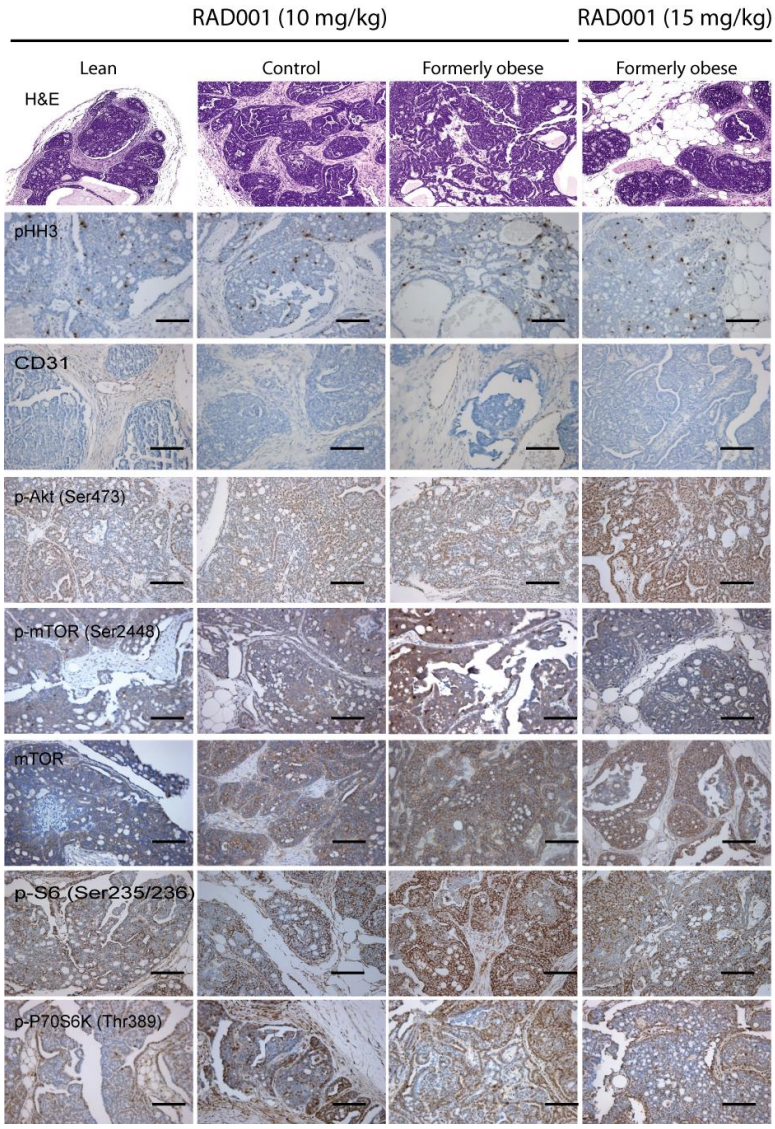


Figure 3.5. MMTV-Wnt-1 tumors from RAD001-treated mice.

RAD001 (10mg/mg) was effective in decreasing protein expression of Akt/mTOR signaling components in CR and control mice. To have a similar effect in tumors from formerly obese mice, a higher dose of RAD001 (15 mg/mg) was necessary.

3.4.6 MOUSE SERUM INCREASES PROLIFERATION AND MIGRATION CAPACITY OF MMTV-E-WNT MAMMARY CANCER CELLS IN VITRO

Our previous animal studies showed a persistent effect of former obesity on postmenopausal mammary tumor growth. We evaluated the ability of the former obese environment to influence mammary tumor growth and performed cell proliferation and migration experiments in vitro. These experiments were aimed to confirm the proliferative effects of former obesity on mammary tumor growth as well as the histological observation of more aggressiveness in tumors from formerly obese mice.

We cultured WG4 mammary tumor cells which are derived from the MMTV-Wnt-1 cell suspension used in our previous animal studies. These cells were exposed to serum extracted from the lean, control, and formerly obese mice at study endpoint. After 48 h of culturing the cells with mouse serum, we found a significant increase in cell proliferation in cells cultured with serum from control (P = 0.05) and formerly obese (P = 0.04) mice when compared to cells growing in serum free conditions (**Figure 3.6A**). Serum from CR mice did not have an effect on cell proliferation. Mammary tumor cell proliferation was not different when comparing cells exposed to formerly obese serum with cells exposed to the control serum.

The processes of cell migration and invasion are integral to cell tumor growth and survival. Deregulated cell motility has been shown to contribute to angiogenesis (155). Furthermore, the correlation between the in vitro migratory potential of tumor cells and the in vivo invasive properties has been previously reported (156). Our animal study showed increased CD31 staining in tumors from control and formerly obese mice. Therefore, to determine the specific effects of the formerly obese environment on mammary tumor cell motility, we conducted a cell migration assay to further investigate the pathological effect of former obesity. In an assay in

which mouse serum was used as the chemoattractant, after a 30 h incubation period, we found a higher number of cells that had migrated through the membrane into the lower compartment with media containing serum from formerly obese mice (**Figure 3.6B**). Very few cells cultured with lean or control serum migrated to the lower chamber. We repeated this experiment using WA4, a more aggressive mammary tumor cell line, and obtained very similar results (not shown).

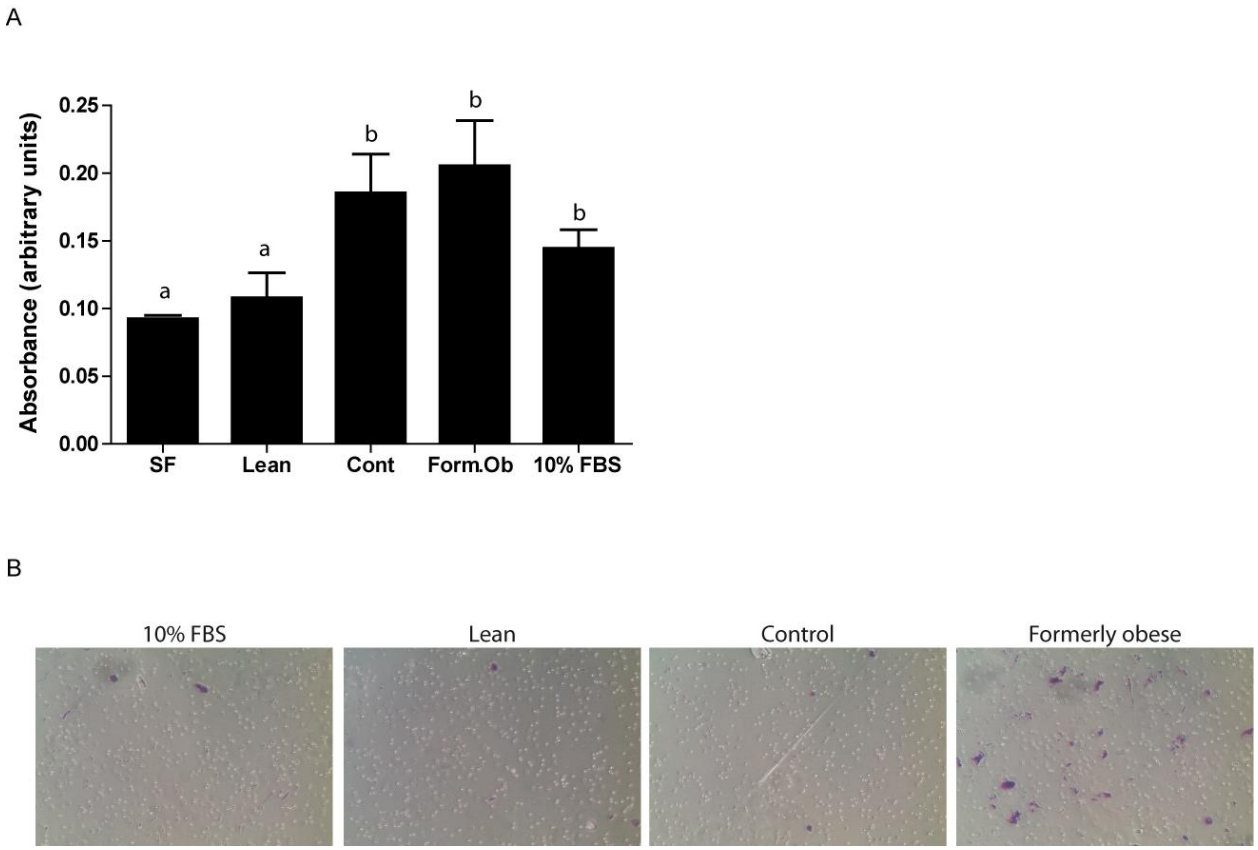


Figure 3.6. Cell proliferation and migration assays of Wnt-1-WG4 mammary tumor cells in vitro.

Effect of serum extracted from lean, control, and formerly obese mice on cell proliferation (A) and migration (B) of WG4 mammary tumor cells. Different letters indicate significant differences ($p<0.05$).

3.5 Discussion

Although various biological mechanisms by which obesity affects breast cancer risk have implicated estrogen and other circulating hormones, it is not known if growth factor signaling pathways will return to basal levels immediately following rapid weight loss. To our knowledge, this is the first study designed to examine the effects of body weight normalization on AKT/mTOR signaling in a DIO mouse model of postmenopausal breast cancer. Our results show that Akt/mTOR signaling remains activated even after rapid weight loss and promotes mammary tumor cell growth. Tumors from formerly obese mice grew faster compared to tumors from the control mice and had increased protein expression of phospho-mTOR, phospho-S6 and phospho-P70S6K.

Previous studies have shown normalization of obesity-associated metabolic markers in high-fat fed mice after a switch to chow. However, since the number of adipocytes is known to remain high in spite of weight loss, it may also continue to exert endocrine effects releasing hormones and cytokines such as TNF-alpha, MCP-1, vefg, and IL-6, that are characteristic of adipose tissue (157, 158). Furthermore, Miller et al., showed that despite normalization of body weight, formerly obese mice continue to have altered gene expression of obesity-related genes, indicating that molecular pathways that control transcription of those genes are still turned on, just as pathways that control tumor growth in our study continue to favor mammary tumor cell growth. In addition, we found that formerly obese mice had increased CD31 staining that correlated with tumor growth. Previous studies have shown that adipogenesis is highly dependent on angiogenic processes that modulate fat mass expansion during obesity (159). Furthermore, adipocytes can secrete angiogenic factors such as vegf that contribute to tumor

vascularization (28). Therefore, better understanding of adipokine function during postmenopausal breast cancer may enable the development of new therapeutic options.

Treatment with mTOR inhibitors is associated with increased Akt (Ser473) activation; a consequence of the mTOR-dependent feedback loop, which may limit some of the antiproliferative effects of RAD001 (160, 161). In addition, enhanced Akt activation seen during obesity may contribute may further decrease the effectiveness of mTOR inhibitors (75). The results from our study showed that Akt/mTOR signaling remains activated even after rapid weight loss and can drive mammary tumor cell growth. Furthermore, we noted that a higher dose of RAD001 was necessary to offset the effect of former obesity on mammary tumor growth suggesting that successful pharmacological interventions in former obesity may require careful dose titration relative to non obese subjects.

AMPK activators, such as metformin, have similar actions to RAD001 and other mTOR inhibitors, resulting in decreased phosphorylation of mTOR and its major substrate S6K1(162). Although both RAD001 and metformin inhibit mTOR, in contrast to RAD001, metformin decreases Akt activation through an AMPK-mediated phosphorylation of IRS-1(Ser789) (162). These data suggest that in the former obese setting there may be anticancer advantages to metformin use, and future studies should focus on comparing mTOR inhibitors with metformin under energy balance conditions.

The goal of these in vitro studies was to determine the effect of the former obese environment on mammary tumor growth. By using serum extracted from these mice we determine global effects of the systemic environment. Using cells derived from the cell

suspension that we injected into the mice allowed us to better compare in vitro studies to the animal studies.

Chapter 4: Obesity-Associated Increases in Serum Insulin-like Growth Factor -1 (IGF-1), Akt/mTOR Signaling, and Mammary Tumor Growth Persist After Recent Weight Loss

4.1 Abstract

The prevalence of obesity among adult American women is 35.5%. Obesity increases breast cancer risk and progression in postmenopausal women. The Akt/mTOR signaling pathway is activated in response to increased levels of obesity-related growth factors, such as insulin-like growth factor (IGF)-1.

We aimed to establish the effects of obesity reversal on postmenopausal mammary tumor growth, growth factors, and cellular signaling pathways. Ovariectomized C57BL/6 mice received control diet (n=17) or a diet-induced obesity (DIO) regimen (n=34) for 17 weeks and body composition was assessed by quantitative magnetic resonance (qMR). Obesity reversal was then initiated by ransomizing 17 of the 34 obese mice to receive the control diet. At week 24, qMR was repeated, and tissues and blood were collected (n=5/group). The remaining mice were orthotopically injected with $5x10^4$ syngeneic MMTV-Wnt-1 mammary tumor cells.

After 17 weeks, obese mice (51.5g ±0.7) weighed more than controls (33.05g ±0.7) ($p<0.05$). By week 24, the obese mice that were switched to control diet (hereafter "formerly obese") displayed body weights and adiposity levels identical to controls and significantly lower ($p< 0.05$) than obese mice. Of the obesity-related hormones and cytokines measured, only circulating IGF-1 levels remained 54% elevated in formerly obese mice (105.16 pg/ml; p=0.05) relative to control (81.83 pg/ml) and were comparable to an increase of 27% in obese mice (126.12 pg/ml; p=0.03). At study endpoint, tumor volume was bigger in obese (1882 mm^3 ± 726)

and formerly obese (2531 mm^3 ± 840) mice compared to control (366.1 mm^3± 305). Phosphorylation of Akt/mTOR signaling components was enhanced in both obese and formerly obese mice. These findings suggest that, recent weight loss is not sufficient to damper tumor proliferative signals consequent to obesity-associated elevations in circulating IGF-1 and Akt/mTOR signaling.

4.2 Introduction

Several studies have associated obesity with an increased risk of postmenopausal breast cancer and recent evidence suggests that adipose tissue-derived hormones and cytokines may provide a link between obesity and breast cancer (23, 44, 163). It has also been shown that obese postmenopausal women with breast cancer have shorter disease-free survival, overall survival, as well as poorer prognosis than non obese women. Evidence from both epidemiological and pre-clinical studies have shown that increased circulating levels of hormones and adipokines including, insulin, leptin, IL-6, IL-10, TNF-alpha, monocyte chemotactic protein-1 (MCP-1), estradiol, and insulin-like growth factor-1 (IGF-1) are characteristic of the obese state and independently influence breast tumor growth (141, 164). In addition, it has been shown that obesity and obesity-related factors like IGF-1 can activate this pathway (75). The Akt/mTOR signaling pathway integrates signals from nutrients and responds to hormones, and growth factors to regulate cell growth and proliferation (79).

Given the obesity epidemic in the US and the obesity-breast cancer connection, strategies to decrease body weight have emerged during the last years. The current clinical evidence suggests that weight loss could be an effective approach to decrease breast cancer risk (11). Both clinical and preclinical studies suggest that weight loss improves circulating levels of obesity-derived

hormones and adipokines known to affect cancer risk (157, 165). However, there is insufficient molecular evidence of how weight loss and changes in hormones and adipokines will affect postmenopausal breast cancer risk.

Mouse mammary tumor virus (MMTV) is strongly associated with the development of spontaneous mammary tumors in mice and is influenced by hormones, particularly estrogen (150). Integration of MMTV proviral DNA ito the Wnt gene family, particularly Wnt-1, contributes to growth and malignant progression of mammary tumors (166). Growing evidence suggests a role for the MMTV and Wnt pathways in the development of human breast cancers (167, 168).

In the present study, we tested the effect of obesity and recent weight loss on body weight in a postmenopausal mouse model of estrogen receptor positive (ER+) breast cancer. After injecting MMTV-Wnt-1 mammary tumor cells (150) into ovariectomized (OVX) C57BL/6 mice, we found that mice that underwent recent weight loss did not have smaller tumors than their obese counterparts. Although weight loss occurred, circulating levels of IGF-1 and subsequent IGF-1/Akt/mTOR signaling remained elevated.

4.3 Materials and Methods

4.3.1 Mice and Diets

All animal protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) and carried out in compliance with all guidelines and regulations. To model the postmenopausal state, six-week-old OVX female C57BL/6 mice were obtained from Charles River Laboratories, Inc. (Animal Production Area, NCI-Frederick,

Frederick, MD), and placed on a chow diet for a week. All mice were individually housed on a 12-hour light/dark cycle and consumed food and water *ad libitum*. Food intake was measured twice a week, and body weights were measured weekly. One week after arrival, mice were randomized to receive either a control diet (n=17; 10 kcal % fat D12450B; Research Diets, New Brunswick, NJ) or a diet-induced obesity regimen (DIO) (n=34; 60 kcal % fat D12492, Research Diets, New Brunswick, NJ) for 17 weeks. At this time, half of the mice fed a DIO diet (n=17) were randomized to receive the control diet. Control, obese, and formerly obese mice were fed their respective regimens for the duration of the study. At week 24, 5 mice per group were fasted and euthanized for baseline tissue collection. Blood was collected by cardiac puncture (150-μL). Whole blood was allowed to clot at room temperature for 30 min prior to centrifugation at 1000 × g for 10 min, and the serum was stored at –80˚C for analysis.

4.3.2 Quantitative Magnetic Resonance

Body composition was measured on all mice at weeks 17 and 24 of diet treatments by quantitative magnetic resonance (qMR) (Echo Medical Systems, Houston TX). Endpoints included lean mass, fat mass, body weight, and percent body fat.

4.3.3 MMTV-Wnt-1 Tumor Cell Injection

At week 24, $5x10^4$ syngeneic MMTV-Wnt-1 mammary tumor cells were injected into the 4^{th} mammary fat pad (MFP) as previously described (152). Briefly, spontaneous mammary tumors from MMTV-Wnt-1 transgenic mice on a C57BL/6 background were mechanically dispersed to form a cell suspension. Viable cells were counted, spun down, and injected in 50 μL of RPMI. Tumor growth was measured 2x/week with skinfold calipers and tumor volume was approximated using the formula $4/3\pi\ r1^2r2^2$(153).

At study endpoint (week 36), the mice were euthanized and the tumors were excised and weighed. Tumors were divided in portions to be formalin fixed, and flash frozen in liquid nitrogen and stored at -80 °C until further analysis.

4.3.4 SERUM HORMONE MEASUREMENT

Serum hormones and cytokines including, leptin, adiponectin, resistin, MCP-1, and estrogen were measured in serum collected at baseline (week 17) and at study endpoint (week 35), using mouse adipokine LINCOplex®Multiplex Assays (Millipore, Inc., Billerica, MA) and analyzed on a BioRad Bioplex 200 analysis system (Biorad, Inc. Hercules, CA). Insulin-like growth factor (IGF-1) concentrations were measured using a Millipore Milliplex Rat/Mouse IGF-1 Single Plex (Millipore, Inc., Billerica, MA; Cat #RMIGF187K).

4.3.5 WESTERN BLOTTING

Mammary tumors from the control, obese and formerly obese mice (random sample of 3/diet group) were homogenized and lysed in RIPA buffer (Sigma, St. Louis, MO) with protease inhibitor tablet (Roche Applied Sciences, Indianapolis, IN) and phosphatase inhibitor cocktails I and II (Sigma). Protein lysates (100µg) were resolved by SDS-PAGE using 6-10% gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked using LI-COR Blocking Buffer for 1 hour at RT (LI-COR Biotechnologies, Lincoln, NE) then incubated with primary antibody (all from Cell Signaling) diluted in Tween-20/TBS (TBS-T) overnight at 4°C. The primary antibodies (and their dilutions) used were against: phospho-Akt (Ser 473) (1:1000), Akt (1:1000), phospho-mTOR (Ser 2448) (1:1000), mTOR (1:1000), phospho-p70/S6K (Thr 389) (1:1000), p70/S6K (1:1000), phospho-S6 (Ser 235/236) (1:1000), and S6 (1:1000). After 4 washes (5 min each) in 0.1% Tween-20/PBS (PBS-T), membranes were

incubated for 45 min at RT in species-specific secondary antibody (LI-COR Biotechnologies) diluted in LI-COR blocking buffer (1:15000). Following 4 washes in PBS-T, membranes were scanned using the Odyssey infrared fluorescent imaging system (LI-COR Biotechnologies). Blots shown are representative of 3 mice per group.

4.3.6 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical (IHC) was performed on a random sample of five tumors per group collected as described (71). Slides were incubated with primary antibodies for the following proteins: total and phosphor-Akt, and phospho-ribosomal S6 and mTOR, phospho-p70S6K, pHH3, and CD31, (Cell Signaling Technology, Danvers, MA), according to protocol.

In all of the samples, proliferating areas were analyzed with the exclusion of necrotic ones. For phospho-S6, phospho-Akt, phospho-mTOR, and CD31, the total number of mitoses in mammary tumors per 3- 20X-magnification fields was counted in a total area of 0.1 mm^2 for each field.

Slides were scanned into the Aperio System (Vista, CA) using the standard ImageScope algorithms for total pixel count and for nuclear immunostaining (phospho-p70S6K, pHH3). The % positive nuclei/cells expression was evaluated according to staining level (1+, 2+, 3+) and distribution.

4.3.7 REAGENTS AND CELL CULTURE

Serum isolated from these mice was used for in vitro studies (1% mouse serum). At study endpoint, serum was aliquoted and stored at -80ºC.

The MMTV-Wnt-1-G4 cell line used here (hereafter called WG4) was previously cloned from a spontaneous mammary tumor from an MMTV-Wnt-1transgenic mouse (152). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine

serum (FBS) (Invitrogen) and penicillin-streptomycin (100 U/ml and 0.1 mg/ml respectively; Sigma-Aldrich) and kept in a humidified atmosphere of 5% CO_2 at 37ºC.

Colony-Forming Cell (CFC) Assay

Cell survival was assessed by colony formation after staining with 1% (w/v) crystal violet (CV) (Sigma-Aldrich) as previously described (26). Briefly, WA4 cells were seeded at a density of 5 x 10^2 cells/well in 6-well plates and incubated for 24 h. After the initial incubation, cells were treated with serum collected from control, obese, and formerly obese mice, washed once in 1X PBS solution (Bio-Rad Laboratories), and returned to complete media without mouse serum. Six to 8 days later, a 1% CV (w/v) staining solution was added to each well for 30 min. Plates were rinsed and inverted for 24 h to dry, and the number of colonies was counted under the microscope.

4.3.8 Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). Oneway analysis of variance (ANOVA) using Tukey's Honestly Significant Difference comparison was used to assess the effects of diet treatment on mean tumor size and serum hormone analyses. For growth proliferation and migration assay, experiments means were compared using Student's t test. Data were examined for extreme values, which were defined as values outside the mean ± three times the standard deviation; extreme values were excluded from analysis as described (169). For all tests SPSS software was used (SPSS Inc., Chicago, IL), and $p \leq 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Body weight and body composition of control, obese, and formerly obese mice.

On a second animal study to evaluate the effects of former obesity on tumor growth, an obese group was added. As expected, obese mice had higher body weight (51.5 g ± 0.7) and fat mass (30.08 g ± 0.4) than control mice (33.05 g ± 0.7) and (12.2 g ± 0.8) respectively at week 17 (**Figure 4.1A**). Percent fat was also higher in obese mice (59.7%) compared to control mice (39.3%) (p<0.001) (**Figure 4.1B**). After weight loss was initiated at week 17, half of the obese mice lost 25% of their body weight (average loss was 12.7 g) relative to the other half that was maintained in the DIO diet (**Figure 4.1C**), hereafter formerly obese. Other body composition parameters including percent fat, lean and fat mass also returned to control levels in these formerly obese mice. These body composition parameters were unchanged in the control or remaining obese mice.

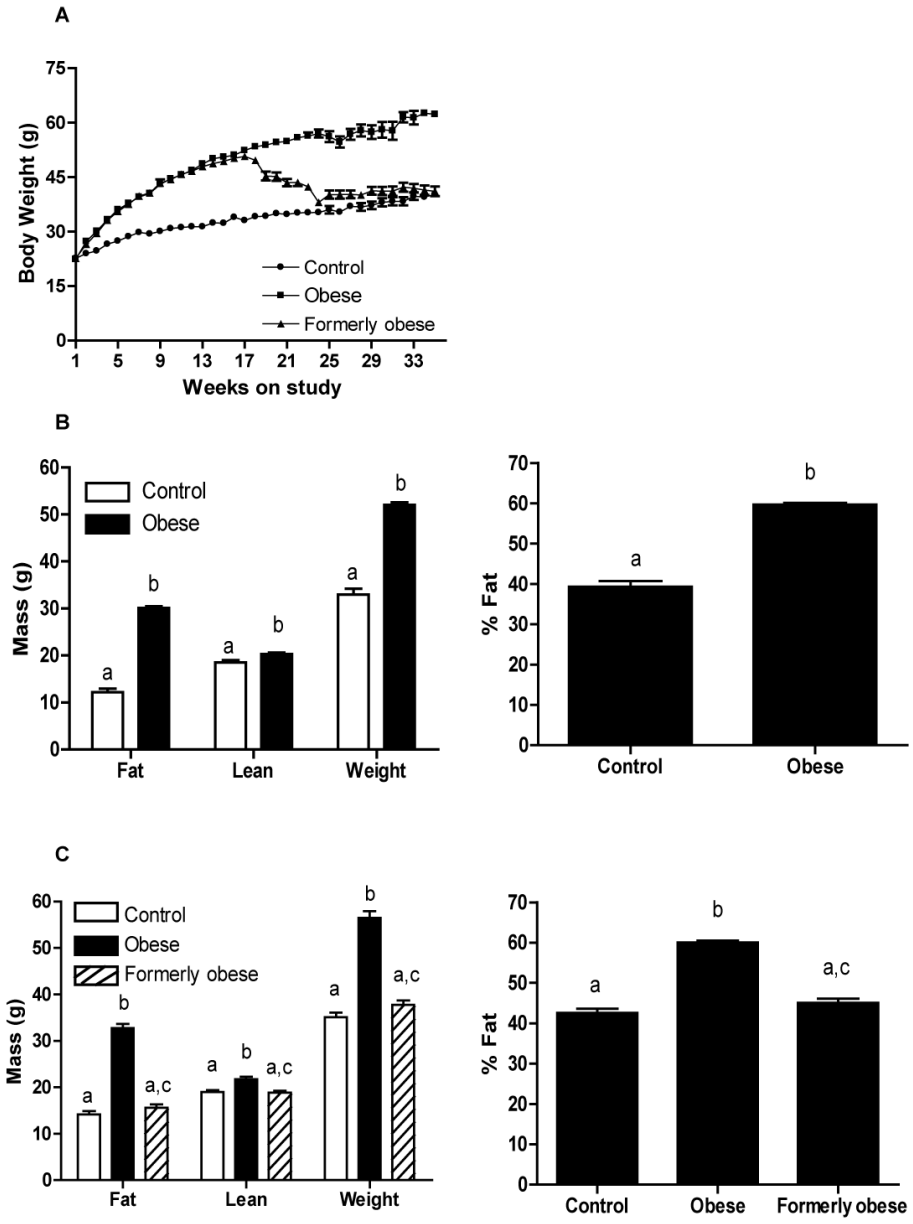


Figure 4.1. Body weight and body composition of C57BL/6 OVX mice.

Body weight for the duration of the study show that DIO mice lose weight after a change to the control diet (A). Body composition at week 17 (B) and 24 (C) of the study.

4.4.2 Effect of weight loss on metabolic markers of obesity.

We have previously shown that obesity-related hormones and cytokines respond to DIO in a mouse model of postmenopausal breast cancer (141). To evaluate the effects of weight loss on obesity-related hormones and cytokines, serum was collected from mice at week 17 (before weight loss) and then again at week 24. Levels of these endocrine markers of obesity were significantly higher in obese relative to controls ($p<0.05$). The reduction in adiposity in the formerly obese mice was accompanied by significantly lower levels of leptin, insulin, and resistin relative to the obese mice, but was not different from control mice. (**Figure 4.2**). Adiponectin was decreased in the obese mice and increased in both control and formerly obese mice. Unlike the reduction in other endocrine markers of obesity, IGF-1 levels remained elevated in formerly obese mice, not different from levels seen in obese mice. In fact, formerly obese mice displayed significantly higher IGF-1 levels than controls ($p=0.03$).

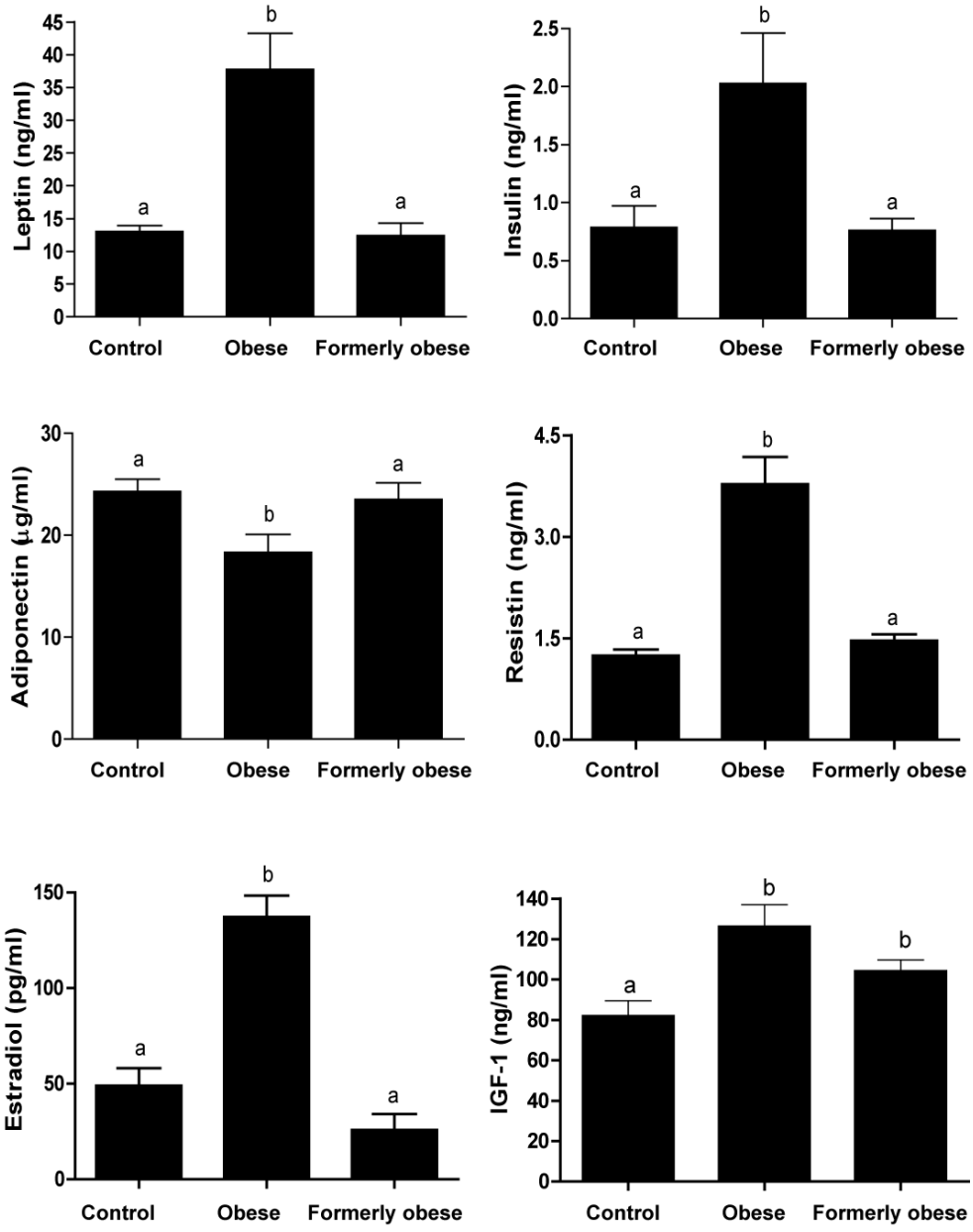


Figure 4.2. Obesity-related hormones and adipokines in C57BL/6 OVX mice at baseline (week 24)

Obesity-related hormones and adipokines of control, obese, and formerly obese mice. Different letters are significantly different from each other.

4.4.3 Increased phosphorylation of Akt/mTOR in mammary fat pads before tumor injection

Because obesity is associated with hormones and growth factors that can activate Akt/mTOR (75), we assessed phosphorylation of Akt/mTOR signaling components in the local environment prior to mammary tumor cell injection. For these analyses, we used mammary fad pads (MFPs) from control, obese, and formerly obese mice (n=5/group) collected at week 24. Increased phosphorylation of Akt (Ser473), mTOR (Ser 2448), S6 (Ser 235/236), and cyclin D1 was observed in both obese and formerly obese mice when compared to control mice (**Figure 4.3**). We further evaluated the MFP for expression of genes known to affect postmenopausal breast tumor growth. Real-time RT-PCR, performed on MFP collected at week 24 and before MMTV-Wnt-1 tumor cell injection, showed that expression of TNF-alpha, insulin receptor substrate-1 (IRS-1), leptin receptor, and cyclin D1 were increased in the formerly obese mice compared to control mice. No difference between obese and control mice were found. However, expression of monocyte chemotactic protein-1 (MCP-1) and TGF-beta was increased in both obese and formerly obese compared to control mice.

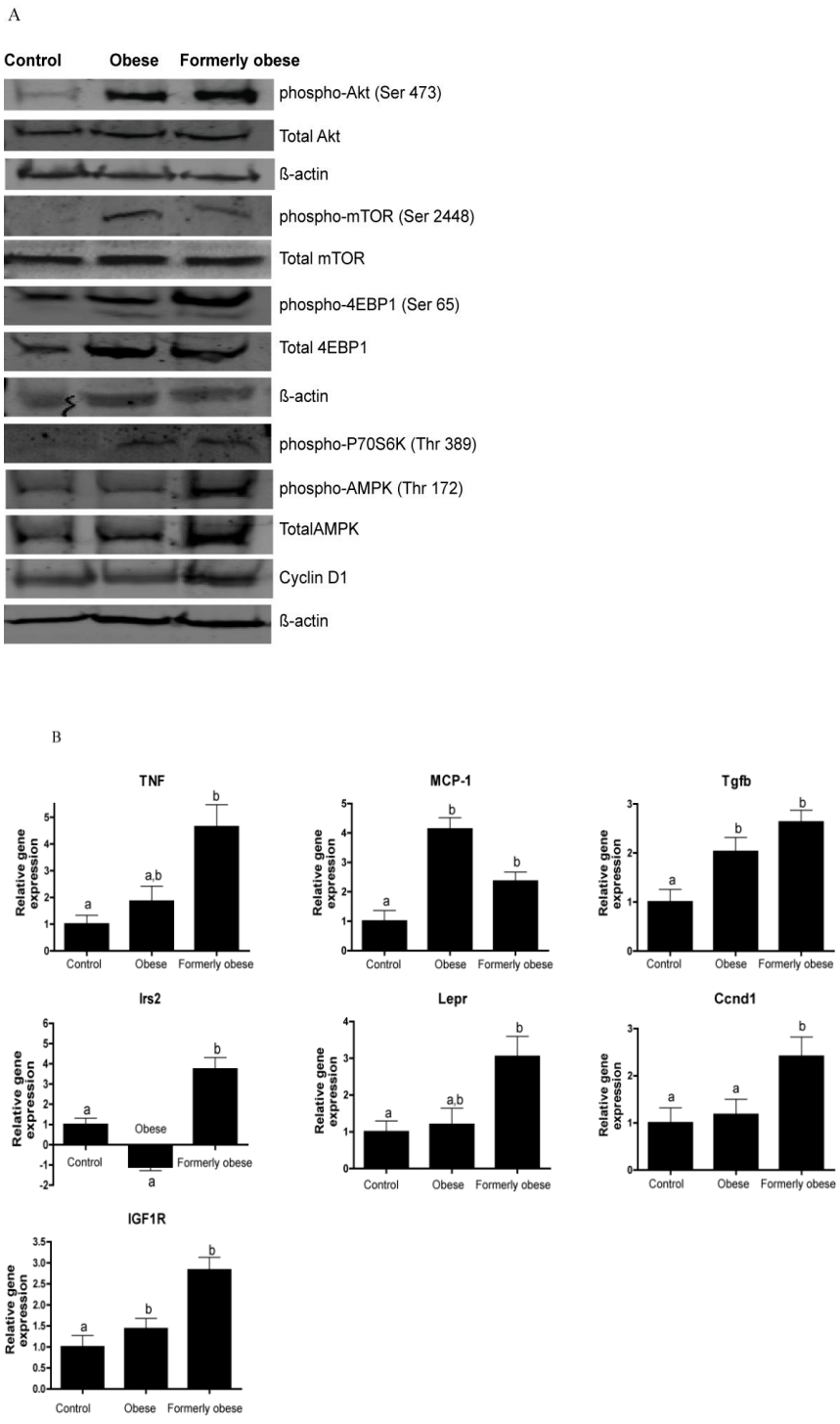


Figure 4.3. Protein and gene expression in mammary fat pad before tumor cell injection.

Baseline activation of Akt/mTOR in the mammary fat pad of C57BL/6 OVX mice (A). Gene expression in mammary fat pads before tumor cell injection (B)

4.4.4 INCREASED TUMOR GROWTH IN BOTH OBESE AND FORMERLY OBESE MICE

Studies from our group have previously reported the detrimental effects of obesity on postmenopausal mammary tumor growth relative to the control diet (141). In the present study, we found that recent weight loss (formerly obese group) did not decrease tumor growth and no difference was found in tumor size between obese and formerly obese mice. At the end of the study, ex vivo tumor measurements confirmed that the tumors from obese (1882 mm^3 ± 726) and formerly obese mice (2532 mm^3 ± 84) were bigger than tumors from the control mice (366 mm^3 ± 305) (p<0.05) (**Figure 4.4 A)**.

Also, tumors showed a predominant tubular pattern with papillary or cystic differentiation. However, the degree of differentiation and the nature of the tumor stroma and vascularization showed qualitative differences between the diet groups. Tumors in the control group consisted of solid masses separated by septi of well vascularized, dense connective stroma. Alternatively, tumors from obese mice were poorly differentiated and in some cases had no pattern of differentiation. In addition, these tumors contained areas of central necrosis and adipocyte infiltration (5/5 tumors). Tumors from formerly obese mice showed septi of connective tissue lobulating the tumor (similar to controls). Also, these tumors had ductal structure with cystic and papillary differentiation and maintained a glandular histology, contrary to tumors from obese mice. A relevant characteristic of tumors from both obese and formerly obese mice was the presence of adipose cells infiltrating the tumor (**Figure 4.4 B)**. Therefore, we looked at tumor protein expression of adipocyte fatty acid binding protein (A-FABP) , which is a 15-kDa protein found in high abundance in the cytosol of adipose cells (170). We found that A-FABP protein expression was increased in tumors from both obese and formerly obese mice, supporting our finding of adipose cells inside these tumors (**Figure 4.5 C**).

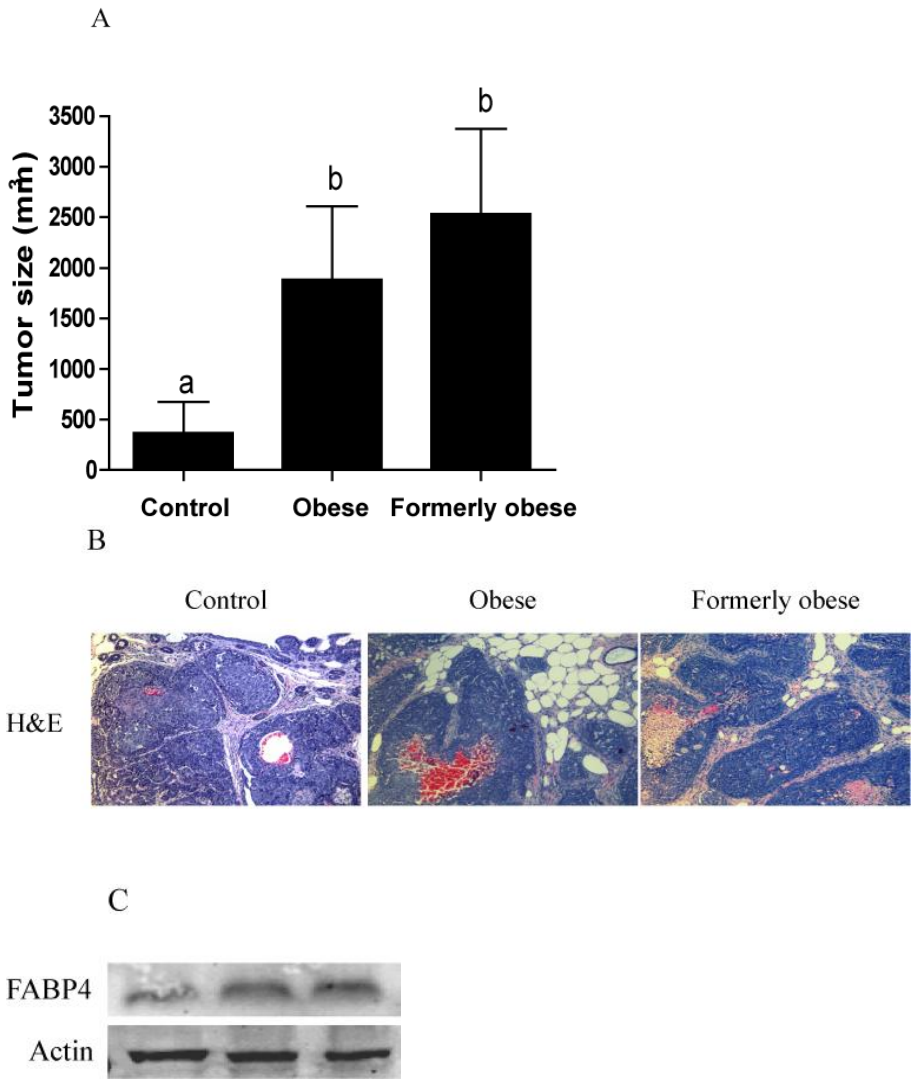


Figure 4.4. MMTV-Wnt-1 mammary tumor in control, obese, and formerly obese C57BL/6 OVX mice.

Obese and formerly obese mice showed increased tumor size relative to controls (A). H&E analyzes showed connective tissue lobulating the tumors of control and formerly obese mice. Tumors from obese and formerly obese mice showed the presence of adipocytes (B) that were confirmed by fatty acid binding protein-4 (FABP4) protein expression (C) .

4.4.5 INCREASED GENE EXPRESSION OF ADIPOCYTE-RELATED FACTORS IN TUMORS FROM OBESE AND FORMERLY OBESE MICE.

Since we saw increased number of adipocytes in tumors from obese and formerly obese mice relative to control, we next examined expression of adipocyte-related genes. Analysis of tumor mRNA expression was done using the mice in the control diet as the reference group. RT-PCR analysis showed that IL-1 β, Vegfa, p21, and S100A9 gene expression was equally increased in tumors from both obese and formerly obese mice. TNF-α gene expression was significantly greater in tumors from obese mice ($p<0.001$) when compared to controls and expression in the formerly obese group was less than obese ($p=0.04$) but higher than control ($p<0.05$) (**Figure 4.5**). IL-6 gene expression was significantly elevated in formerly obese ($p=0.004$). No difference was found in IL-6 gene expression between obese and control mice. IL-10 was increased in tumors from both obese and formerly obese mice, nevertheless, expression was higher in formerly obese than obese mice ($p<0.001$). S100A9, a macrophage marker, and Vegfa were significantly increased in tumors from both obese and formerly obese mice when compared to tumors from the controls ($p<0.001$). However, expression of monocyte chemotactic protein-1 (MCP-1) was no different between tumors from obese and control mice, it was higher in tumors from formerly obese and this increase was different than from controls ($p<0.05$).

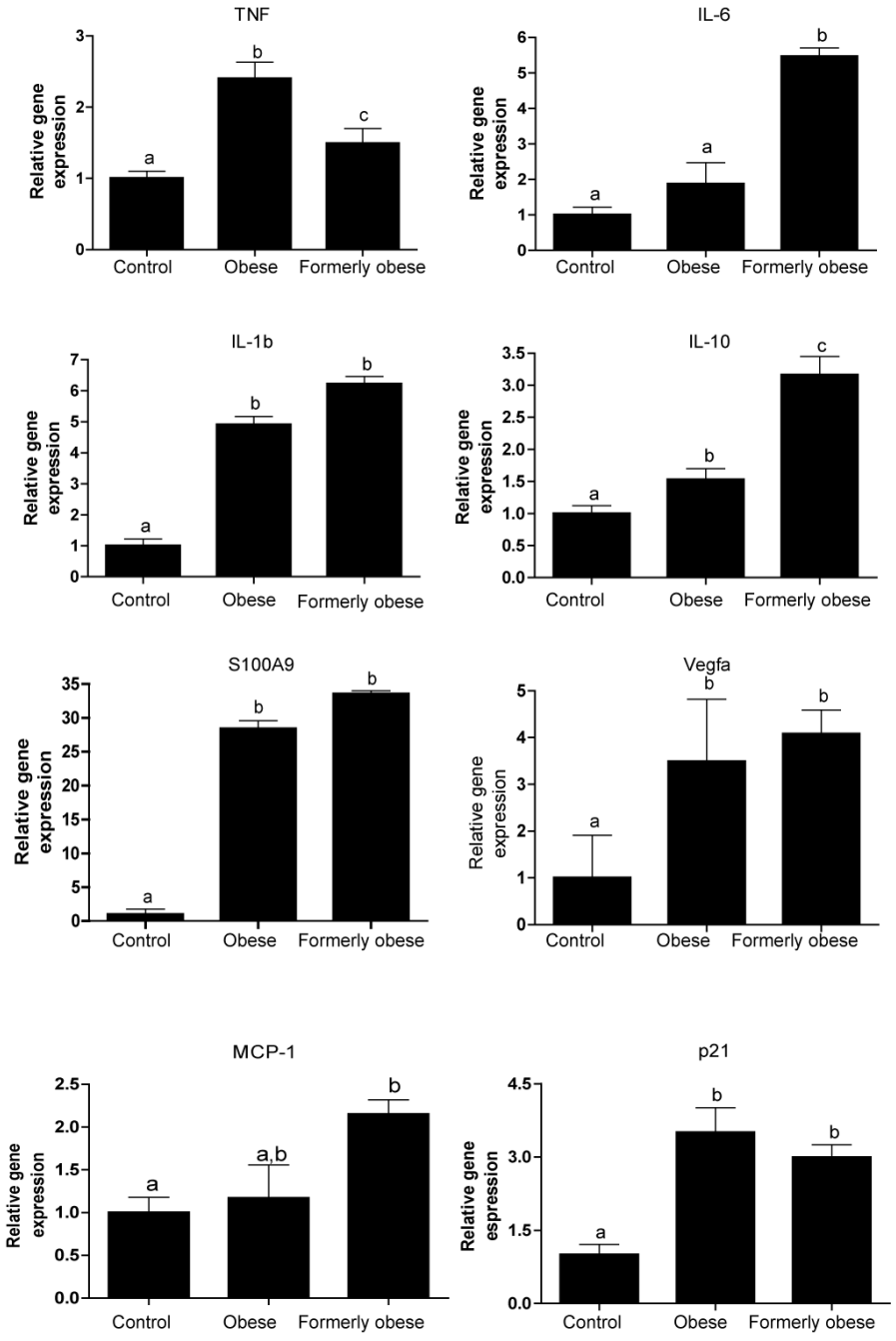


Figure 4.5. Endpoint gene expression in tumors from control, obese, and formerly obese C57BL/6 mice.

Obese and formerly obese mice show increased expression of growth factor, inflammation, and cell cycle- related genes.

4.4.6 AKT/MTOR SIGNALING PATHWAY IS ACTIVATED IN TUMORS FROM OBESE AND FORMERLY OBESE MICE.

Previous studies have shown that IGF-1 can activate Akt/mTOR signaling (29). Because circulating levels of IGF-1 were significantly increased in serum from obese and formerly obese mice compared to the control mice, in the current study, we looked at Akt/mTOR signaling. All dietary groups showed Akt protein expression. Inmunoblotting showed that phosphorylation of Akt (Ser 473) was increased in tumors from both obese and formerly obese mice as compared to control mice (**Figure 4.6**), as well as other key components of the Akt/mTOR signaling pathway including 4EBP-1 (Ser 65), S6 (Ser235/236), p70S6K (Thr 389), and the mTOR-regulated protein, cyclin D1.

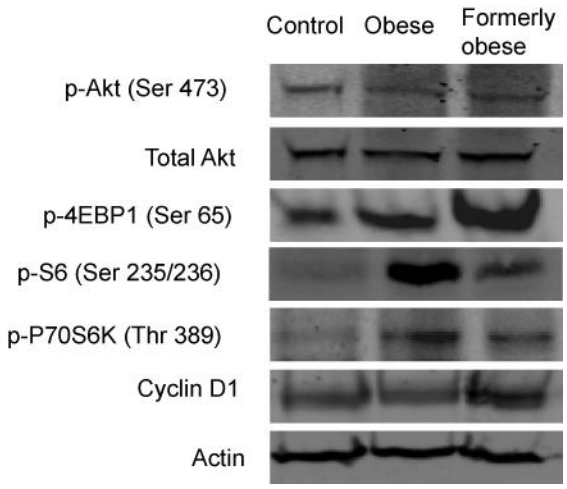
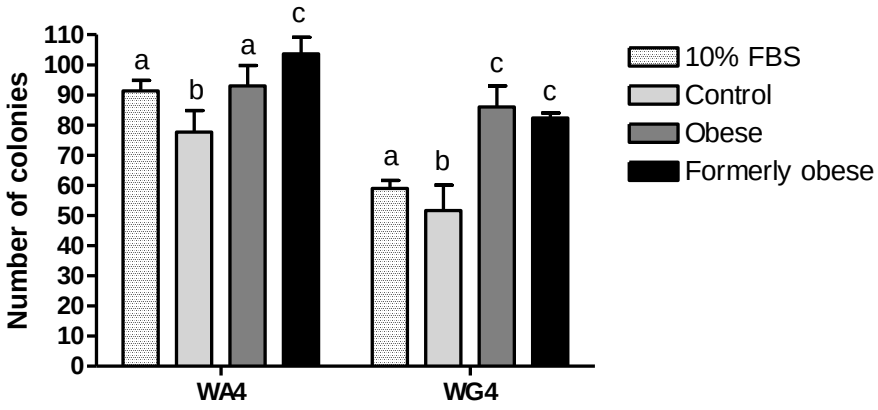


Figure 4.6. Increased Akt/mTOR signaling at study endpoint in MMTV-Wnt-1 mammary tumors.

Tumors from obese and formerly obese mice displayed increased phosphorylation of Akt/mTOR signaling components.

4.4.7 SERUM FROM FORMERLY OBESE MICE INCREASES COLONY FORMATION MMTV-WG4 MAMMARY CANCER CELLS IN VITRO.

Because thus far differences in tumor growth were associated with Akt/mTOR signaling in response to serum levels of IGF-1, we examined the effects of serum from these mice at week 24 directly on MMTV-Wn-1-A4 and G4 cancer cells in an in vitro system. It has previously been demonstrated that increased secretion of hormones and cytokines in response to obesity, promotes breast tumor cell proliferation (171). After 5 days of culture, both the number and size of colonies was higher in cells cultured with serum from obese and formerly obese mice than in cells cultured with serum from control mice (**Figure 4.7**). Cell colony formation was not different between cells cultured with serum from control mice and 10% FBS ($p>0.05$).



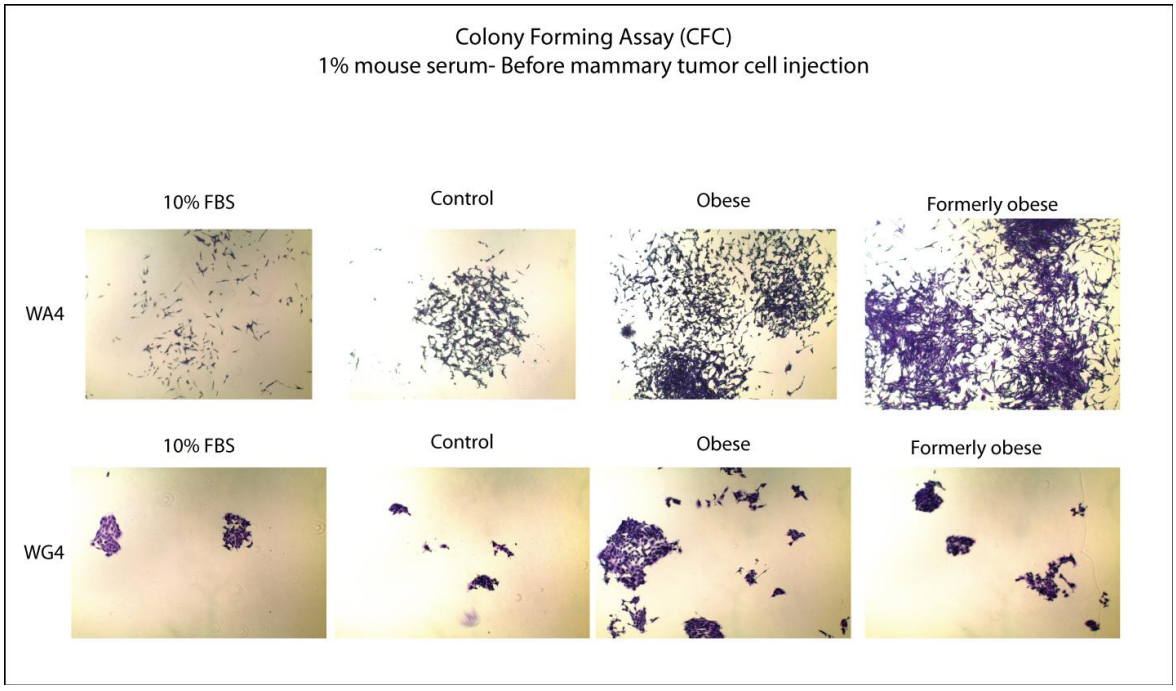


Figure 4.7. Colony forming cell assay in WG4 cells.

After 5 days of tissue culture, A4 and G4 cells cultured with serum from obese, and formerly obese serum show increased number and size of cell colonies formed.

4.5 Discussion

The aim of this study was to consolidate evidence that Akt/mTOR signaling pathway is a likely target to decrease the effects of obesity and former obesity on MMTV-Wnt-1 postmenopausal mammary tumors.

There are limited clinical and preclinical studies evaluating the effect of weight loss on postmenopausal breast cancer risk, but there is an indication of a decreased risk for breast cancer among women who have lost weight (11). However, these data are inconclusive and most of these studies have not been able to evaluate metabolic and molecular changes that follow weight loss and its effects on breast cancer progression (11). In this study we looked at the effects of both obesity and former obesity on postmenopausal mammary tumor growth. We used a mouse model of postmenopausal breast cancer in which ovariectomy closely resembles menopause in women, which is characterized by decreased levels of circulating estrogen, decreased bone mineral density, and cessation of the estrous cycle (151). Our results showed that mice fed a DIO diet quickly gained weight and became obese compared to the mice fed a control diet. Once half of the obese mice were switched to the control diet, they lost the weight and became no different from never obese control mice. Evaluation of body composition parameters and metabolic markers of obesity confirmed that after losing body weight, formerly obese mice were not different from mice in the control diet, except that serum IGF-1 levels remained high in the formerly obese mice.

Adipose tissue is a major endocrine organ that secretes hormones and adipokines known to affect breast cancer growth (23, 44, 171). These adipokines include leptin, resistin, and insulin. Studies have shown that circulating levels of leptin, insulin, and resistin decrease after weight loss; also, a decrease in fat cell size is usually observed (172, 173). On the other hand, obese individuals

have decreased levels of adiponectin which usually increase with weight contributing to sensitize tissues to insulin action (174). In support of the epidemiological data, here we show that in formerly obese mice, levels of these adipokines and hormones such as estrogen and insulin were also returned to the levels of never obese control mice. In formerly obese mice, only circulating IGF-1 levels were not different from obese mice and were higher relative to IGF-1 levels in the control mice.

The importance of microenvironmental alterations in shaping tumor development and progression has been demonstrated before (175). Furthermore, cell to cell and cell-microenvironment interactions can affect the proliferation, survival, differentiation, and invasive capacity of mammary epithelial cells. However, there is not enough epidemiological data indicating normalization of IGF-1 levels following weight loss. IGF-1 has mitogenic effects that can activate signaling pathways including Akt/mTOR signaling, therefore promoting cell survival and proliferation in the microenvironment. In our study, Akt/mTOR signaling was activated before and after tumor injection in both obese and formerly obese mice. Akt/mTOR basal activation in the MFP of obese mice did not disappear in the formerly obese MFP. This result suggests that molecular-driven alteration in the obese state are not reverted rapidly after weight loss. Both obese and formerly obese mice had increased serum IGF-1 levels relative to mice in the control diet, indicating that IGF-1 is a causative agent in the progression breast cancer at least in our postmenopausal mouse model. In our study, we found basal Akt/mTOR activation in MFP from obese mice and this effect did not disappear in the formerly obese MFP.

In our study, tumor size was not different between obese and formerly obese mice and both of these groups had tumors bigger than then control. This is particularly surprising considering that, at the time of tumor cell injection formerly obese mice were no different from

control mice in terms of body weight, body composition, and metabolic markers of obesity, with the exception of IGF-1. Akt/mTOR signaling pathway activation was observed in both obese and formerly obese mice.

Our results suggest relevant clinical considerations in the context of former obesity and breast cancer therapy, such as that successful pharmacological intervention in former obesity studies may require a careful titration of RAD001 relative to nonobese counterparts. However, more studies need to be done before this finding can be applicable to the clinic setting as there is lack of consensus to estimating pharmacokinetic parameters of several drugs when excess body weight is an issue (176). Furthermore, these findings suggest that the growth-enhancing effects of obesity on mammary tumor may persist even after rapid weight loss and suggest that a combination of dietary and pharmacologic interventions, such as mTOR inhibitors, may be required to break the obesity-cancer link.

Chapter 5: Anti-tumor Effects of Ursolic Acid in a Mouse Model of Postmenopausal Breast Cancer

5.1 Abstract

Over the past two decades, bioactive natural compounds have been shown to be a plausible adjunct to the treatment of breast cancer, the second leading cause of cancer death among American women. The present study was designed to investigate the effects of ursolic acid (UA), a pentacyclic triterpene found in many foods and herbs, in a model of postmenopausal breast cancer. Ovariectomized C57BL/6 mice (n=40) were randomized to receive control diet (AIN-93G) or diet supplemented with UA at one of three doses (w/w): 0.05%, 0.10%, or 0.25% (~54, 106, or 266 mg/kg body weight/d, respectively). After 3 weeks, syngeneic MMTV-Wnt-1 mammary tumor cells were injected in the mammary fat pad, and mice continued on their respective diets for five more weeks. All UA doses decreased tumor cell proliferation, as assessed by Ki67 immunostaining; nevertheless, UA at 0.10% was most effective in inhibiting tumor take and decreasing tumor final tumor size. Modulation of Akt/mTOR signaling and induction of apoptosis appeared to mediate these effects on tumor growth. UA potently disrupted cell cycle progression and induced necrosis in a clonal MMTV-Wnt-1 mammary tumor cell line in vitro. This study supports the potential of UA as an antitumorigenic agent.

5.2 Introduction

Naturally occurring agents are an alternative for improving cancer therapeutics. Research has provided evidence that diets rich in fruits and vegetables provide protective effects against the risk of several types of cancers (177, 178); this anti-carcinogenic effect may be due to bioactive food components directly targeting pathways that become aberrant during cancer growth (179). However, the role of many bioactive food components in breast cancer is not yet fully understood. Therefore, studies investigating specific components in different cancer models are an excellent approach to improving cancer therapeutics, particularly for postmenopausal breast cancer, the most common noncutaneous cancer in American women (1).

A significant proportion of the drugs currently used to treat cancer are either natural products or compounds based on natural products (2), proving them to be an effective approach for prevention and treatment of many cancers or when other therapies have failed (87, 88). Ursolic acid (UA) is a pentacyclic triterpene member of the cyclosqualenoid family, which is ubiquitous in the plant kingdom and found in many foods and herbs, such as apple, cranberry, rosemary, and oregano (180). UA has been shown to inhibit several cancers and to improve glucose tolerance in high-fat-fed mice, and it can also affect cell growth, differentiation, and apoptosis in vitro (90, 92, 181). Secondary metabolites of triterpenes, such as camptothecin and paclitaxel, are currently used as chemotherapeutic agents (93).

The phosphatidylinositol 3’-kinase (PI3K)/Akt and MAPK cellular signaling pathways are important in cancer development and progression. Bioactive food components have been shown to affect these pathways, as well as others, both in vitro and in vivo (94, 97, 182, 183). UA can inhibit growth of endometrial cancer cells, and this effect is mediated by inhibition of

both of these signaling pathways (98). However, studies regarding UA effects are limited, with little indication of the impact that UA may have on molecular pathways activated during postmenopausal breast cancer. Nevertheless, given the current evidence of its anticancer properties and low toxicity (99), further study of the possible effects of UA on inhibiting mammary tumor growth in a postmenopausal model is warranted.

The aim of this study was to determine the effect of a dietary dose-range of UA on a mouse model of postmenopausal breast cancer and gain better understanding of the possible mechanisms of action. We used ovariectomized C57BL/6 mice as a model of the postmenopausal state, which were injected with syngeneic tumor cells from the MMTV-Wnt-1 transgenic mouse. Similar to postmenopausal women, ovariectomized mice experience increased weight gain and decreased bone mineral density (184), while, like many breast cancers, MMTV-Wnt-1 mouse tumors express estrogen receptors (185). Results from this study will facilitate a better understanding of UA effects on breast cancer.

5.3 Materials and Methods

5.3.1 Mice and Diets

All animal protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and carried out in compliance with all guidelines and regulations. Six-week-old ovariectomized female C57BL/6 mice (n=40) were obtained from Charles River Laboratories, Inc. (Animal Production Area, NCI-Frederick, Frederick, MD), and placed on a chow diet. All mice were individually housed on a 12-hour light/dark cycle, and consumed food and water *ad libitum*. Food intake for all groups was measured twice a week, and

body weights were measured weekly. One week following arrival, mice were randomly assigned (n=10 per group) to receive for 8 weeks either a control diet (AIN-93G) or that same diet supplemented with the triterpenoid UA at three different concentrations: 0.05%, 0.10% or 0.25% (w/w in the diet). Experimental diets were formulated by BioServ, Inc. (Frenchtown, NJ) and contained a natural extract of UA from European cranberries (Sigma-Aldrich). Control and UA-supplemented diets contained comparable nutrient levels. There have been few in vivo studies evaluating UA specifically; therefore, we selected our three dose levels to bracket the usual dose of phytochemical tested (186-188).

After 3 weeks on the diet and at the end of the study (week 8), mice were fasted for 10 hours with ad libitum access to water prior to collection of blood samples via the retro-orbital sinus (after week 3) or cardiac puncture (week 8). Whole blood was processed and serum stored as previously described (189).

5.3.2 MMTV-WNT-1 TUMOR CELL INJECTION

After 3 weeks on the diet treatments, mice were injected with syngeneic MMTV-Wnt-1 mammary tumor cells as previously described (184) and followed for 5 more weeks. Briefly, spontaneous mammary tumors from MMTV-Wnt-1 transgenic mice on a C57BL/6 background were mechanically dispersed to form a cell suspension and viable cells counted. All mice were injected with $5x10^4$ cells/mouse in the 9^{th} mammary fat pad as previously described, with the exception that no incision was made to visualize the mammary fat pad. Mice were palpated daily, and palpable tumors were measured 3 times a week with electronic skinfold calipers. Tumor volume was approximated using the formula $4/3\pi\ r_1^2 r_2$ (153). At study endpoint (week 8 on diets; 33 days after tumor cell injection), the mice were euthanized and the tumors were

excised and weighed. Tumors were randomly assigned to be either formalin fixed or to be flash frozen in liquid nitrogen and stored at -80 °C until further analysis.

5.3.3 Immunohistochemical Analysis

Immunohistochemistry (IHC) was performed on a random sample of four tumors per group collected as described (190). Slides were incubated with primary antibodies for the following proteins: Ki67, cleaved-caspase-3, total and phospho Akt, and phospho ribosomal S6 (Cell Signaling Technology, Danvers, MA), according to protocol.

5.3.4 Serum Hormone Measurement

Serum insulin, monocyte chemotactic protein-1 (MCP-1), and insulin-like growth factor (IGF-1) concentrations were measured as previously described (184).

5.3.5 Western Blot Analysis

Tumor samples were thawed on ice, and tumor lysates were prepared by homogenizing tumors in 10 ml lysis buffer (1M HEPES (pH 7.5), 5M NaCl, 500 mM EDTA, 10% TritonX, 0.5 g sodium deoxycholate, 20 mM sodium fluoride, 10 mM sodium molybdate, 10 mM sodium vanadate) containing Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN). The protein concentration of the supernatant was measured on a BioPhotometer (Eppendorf, Westbury, NY). Tumor lysates were diluted at 1:1 with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) and loaded on 4-20% SDS-PAGE gradient gels, for separation by electrophoresis. The proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories), and membranes were blocked for 1 hour with LI-COR Blocking Buffer (LI-COR Biotechnology, Lincoln, NE). For immunodetection, the membranes were probed overnight

with antibodies (all from Cell Signaling) specific for proteins downstream of insulin signaling: pAkt(Ser473), pS6(Ser235/2365), pMAPK p44/42, phospho-IGF-1 receptor β (Tyr1135/1136)/insulin receptor-β (Tyr1150), and cyclin D1, or for β-actin. The membrane was then washed with phosphate-buffered saline-0.1% Tween-20 (PBS-T) and incubated with IRDye secondary antibodies (LI-COR Biotechnology) for 45 minutes. Fluorescent activity was detected and quantified using the LI-COR *Odyssey* Infrared Fluorescent Imaging System (LI-COR Biotechnology).

5.3.6 Reagents and Cell Culture

UA (Sigma-Aldrich, St. Louis, MO) was prepared as a 100 mM stock solution in dimethylsulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA) and stored at -20 ºC. For all experiments, final concentrations of UA were prepared by diluting the stock with RPMI-1640. Control cultures received the carrier solvent (0.1% DMSO).

The MMTV-Wnt-1-A4 cell line used here (hereafter called WA4) was previously cloned from a spontaneous mammary tumor from an MMTV-Wnt-1transgenic mouse (191). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin-streptomycin (100 U/ml and 0.1 mg/ml respectively; Sigma-Aldrich) and kept in a humidified atmosphere of 5% CO_2 at 37ºC.

5.3.7 Cell Viability Assay

WA4 cells were seeded at a density of 3 x10^3 cells/well on 96-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). After 48 h incubation to allow cells to adhere, confluent cells were treated with UA (0-50 μM) for 24 h at 37 ºC. Calcein AM (2 μM in PBS) (Biotium, Inc., Hayward, CA) was added to each well and incubated at 37 ºC for 30 min. Cell

viability was determined by measuring the fluorescence of calcein in live cells according to the manufacturer's protocol. The amplitude of the fluorescent signal is proportional to the number of living cells.

5.3.8 COLONY-FORMING CELL (CFC) ASSAY

Cell survival was assessed by colony formation after staining with 1% (w/v) crystal violet (CV) (Sigma-Aldrich) as previously described (192). Briefly, WA4 cells were seeded at a density of 5 x 10^2 cells/well in 6-well plates and incubated for 24 h. After the initial incubation, cells were treated with UA (0-50 μM) for 24 h at 37 ºC, washed once in 1X phosphate buffered saline (PBS) solution (Bio-Rad Laboratories), and returned to complete media without UA. Six to 8 days later, a 1% CV (w/v) staining solution was added to each well for 30 min. Plates were rinsed and inverted for 24 h to dry, and the number of colonies consisting of more than 50 cells were counted under the microscope.

5.3.9 CELL CYCLE ANALYSIS

To determine UA effects on cell cycle, 1x10^6 WA4 cells were plated in complete culture medium in T25 flasks; cells were allowed to become confluent and then incubated with UA (0-50 μM) for 24 h. After 24 h, cell cycle analysis was performed using propidium iodide (PI). For PI staining, cells were fixed for 60 min at room temperature in a 1% (w/v) solution of paraformaldehyde (Fischer Scientific, Fair Lawn, NJ) in PBS and stored overnight at -20 °C. Cells were then washed in PBS and resuspended in 400 μl of PI solution (50 μg/ml PI, Sigma-Aldrich) prior to flow cytometry analysis using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). A total of 50,000 gated events were collected using an FL2 area x FL2 width

histogram. Analysis was performed on the resultant FL2 area histogram using the MODFIT-LT (Verity Software House, Topsham, ME) analysis software.

5.3.10 Apoptosis Analysis

Confluent WA4 cells were treated with UA (0-50 μM) for 24 h before apoptosis analysis. The apoptotic index in these cells was identified by Annexin V-FITC apoptosis detection kit (BD Biosciences), which detects the percentage of cells actively undergoing apoptosis, in combination with PI to distinguish viable from nonviable cells. Analysis was performed using the FACSCalibur flow cytometer (BD Biosciences), and linear emission of Annexin V-FITC and PI was collected using FL1 (FITC) x FL2 (PI) detectors in logarithmic mode. Events were gated based on the dual parameter light scatter histogram (FSC x SSC). Cells positive for Annexin V-FITC and negative for PI are apoptotic. Cells positive for both Annexin V-FITC and PI are considered end-stage apoptosis, necrotic, or dead. Cells negative for both Annexin V-FITC and PI are viable and with no measurable apoptosis. Analysis was performed using FlowJo (Ashland, OR) flow cytometry analysis software.

5.3.11 Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed between UA dietary groups (i.e., 0.05%, 0.1%, or 0.25%), except as noted. For all tests, SPSS software was used (SPSS Inc., Chicago, IL), and $P < 0.05$ was considered statistically significant. Statistical analysis of the tumor-free survival curves was performed by applying the log-rank test on the survival curves with the end point set as "time to tumor." One-way analysis of variance (ANOVA) using Tukey's Honestly Significant Difference comparison was used to assess the effects of diet on mean tumor size and serum hormone analyses. Data

were examined for extreme values, which were defined as values outside the mean ± three times the standard deviation (Peirce's criterion); extreme values were excluded from analysis as described (169).

5.4 Results

5.4.1 DIETARY URSOLIC ACID DECREASES THE NUMBER OF PALPABLE TUMORS IN VIVO

Ovariectomized C57BL/6 mice consumed control or UA diet treatments throughout the 8-week study. No toxicity was observed, and food consumption and body weights were not affected by the presence of UA in the diet (w/w) at 0.05% (~54 mg/kg body weight/d), 0.10% (~106 mg/kg body weight/d) or 0.25% (~266 mg/kg body weight/d) (**Figure 5.1**). After 3 weeks on dietary treatments, mice were then injected with dispersed MMTV-Wnt-1 tumor cells, and tumor size was monitored for 33 days. We observed a rapid onset of palpable tumors in the control group as early as 21 days post tumor cell injection (**Figure 5.2**); two tumors were very fast growing, and the mice had to be euthanized at day 27. While the lowest dose of UA (0.05%) had no effect on tumor size or weight, mice consuming the intermediate 0.10% UA dose had significantly smaller tumors than control throughout the study and final tumor weights ($p<0.05$) The 0.10% UA dose also decreased development of palpable tumors; remarkably, 4/10 mice (40%) in this group had no evidence of a tumor at end point. Moreover, 3/10 mice (30%) and 2/10 mice (20%) in the 0.05% UA and 0.25% UA dose groups, respectively, did not develop tumors, while 10/10 mice in the control group (100%) developed palpable tumors. The higher UA dose (0.25%) decreased palpable tumor size significantly compared to control up to 28 days after injection, but final tumor weights at day 33 did not differ significantly from control. Therefore no dose-response was observed in this study.

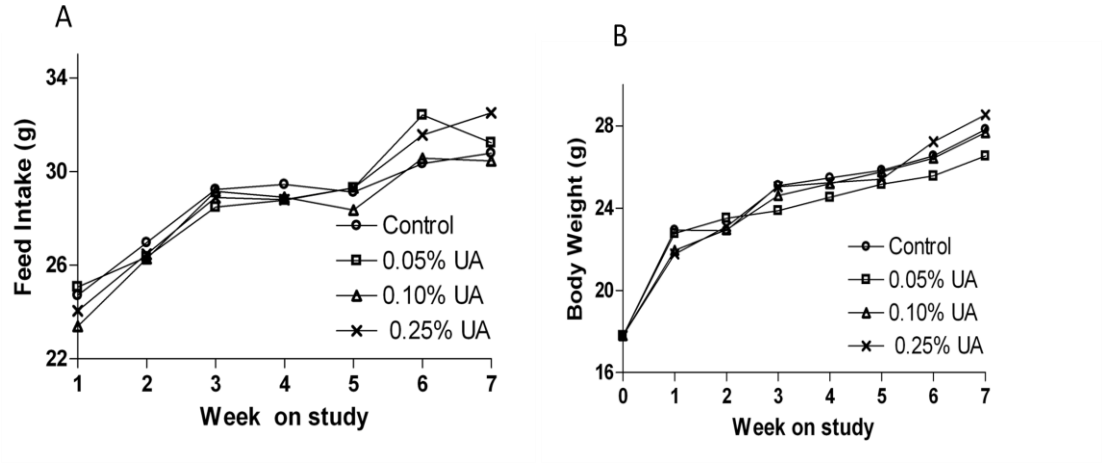


Figure 5.1. Food intake and body weight.

(A) Mean weekly food consumption. Mice were randomly assigned (n=10/group) to receive either a control diet (AIN-93G) or that same diet supplemented with UA at three different doses (w/w): 0.05% (~54.3 mg/kg body weight/d), 0.10% (~105.7 mg/kg body weight/d) or 0.25% (~265.7 mg/kg body weight/d). Food intake was measured weekly and was not affected by the presence of UA in the diet. (B) Supplementary Figure 2. Mean weekly body weights. Body weight was measured weekly and was not affected by the presence of the UA in the diet (n=10/group).

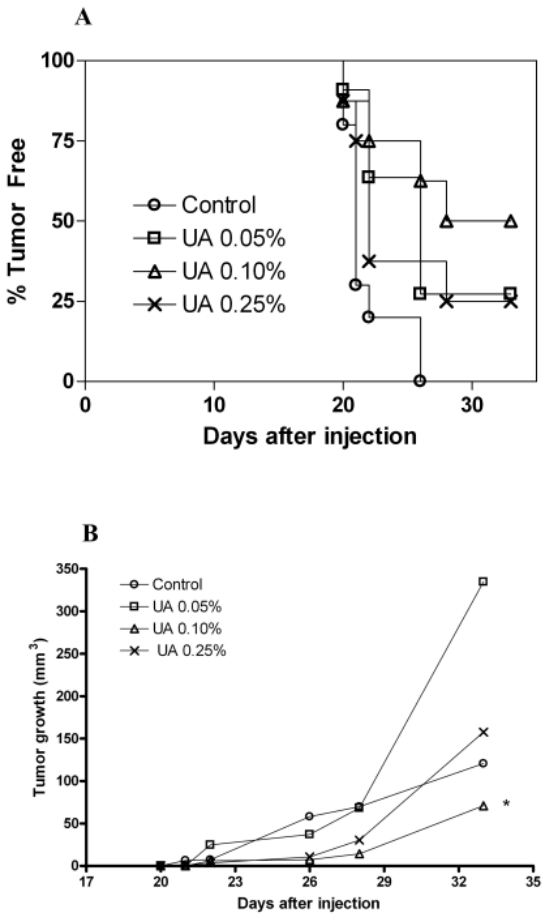


Figure 5.2. MMTV-Wnt-1 mammary tumor growth after UA dietary treatment.

UA decreases mammary tumor size in vivo. Ovariectomized C57BL/6 mice were treated with an ursolic acid-supplemented diet (0.05%, 0.10%, or 0.25% w/w) for 8 weeks. After 3 weeks on the diets, the mice were injected in the mammary fat pad with syngeneic MMTV-Wnt-1 mammary tumor cells. Percent tumor free (mice without palpable tumors) is represented in (A); 0.10% UA significantly delayed the appearance of palpable tumors ($p<0.05$). Mammary tumor size and final tumor weight were significantly lower ($p<0.05$) in mice receiving 0.10% UA diet (B,C). *Significantly different from control at $p<0.05$.

5.4.2 Evaluation of proliferation, apoptosis, and Akt pathway activation in mammary tumors by IHC

Because our study demonstrated a significant decrease in tumor size in mice treated with 0.10% UA, we examined molecular changes by IHC (**Figure 5.3**). Hematoxylin and eosin staining (H&E) did not reveal any differences in tumor grade between the treatment groups. UA treatment induced a dose-dependent decrease in cellular proliferation, as measured by Ki67 staining, while we observed elevated cleaved-caspase-3 expression, a marker of apoptosis, in the mice receiving 0.10% UA only. In further confirmation of western blotting data (shown later), UA treatment induced a dose-dependent decrease in Akt phosphorylation and activation (Ser473), with little change in total Akt expression across treatment and control groups. Additionally, we saw a marked decrease in pS6 expression in all UA treatment groups.

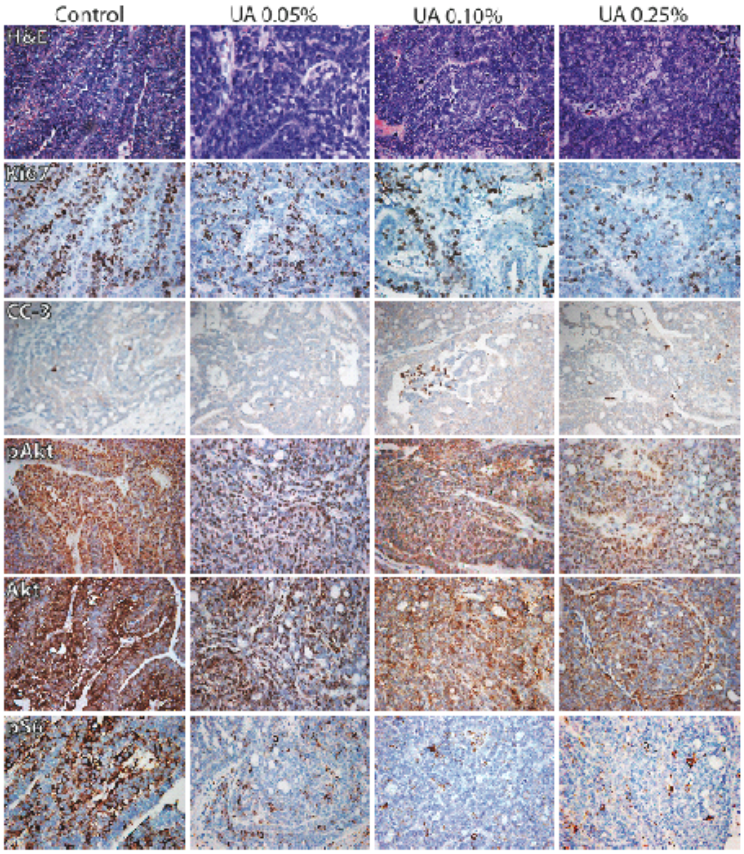


Figure 5.3. UA diet treatment affects proliferation, apoptosis, and Akt/mTOR signaling in MMTV-Wnt-1 mammary tumors.

Tumors from ovariectomized C57BL/6 mice were collected at study endpoint (Week 8), formalin-fixed, paraffin-embedded, and analyzed by IHC. UA treatment induced a dose dependent decrease in cellular proliferation, as measured by Ki67 staining, and UA (0.10%) increased cleaved-caspase-3 protein expression, indicating increased apoptosis. UA treatment induced a dose-dependent decrease in Akt phosphorylation and activation (Ser473), with little change in total Akt expression across treatment and control groups. Additionally, UA treatment dramatically decreased pS6 activation, an mTOR effector involved in translation initiation.

5.4.3 URSOLIC ACID EFFECTS ON CIRCULATING LEVELS OF INSULIN, MCP-1, AND IGF-1

We next evaluated growth factor and inflammatory markers, including insulin, MCP-1, and IGF-1, in serum of these mice. Insulin and MCP-1 levels were measured after 3 weeks on the diets, just before tumor cell injection (pre-tumor), and at study endpoint (post-tumor). After 3 weeks on the diet treatments, insulin levels in the group receiving 0.25% UA were significantly lower (p=0.02) than in the control group, whereas at study endpoint, insulin levels in the 0.25% UA group were significantly higher (p<0.001) than controls (**Figure 5.4**). After 3 weeks on the diet treatments, 0.25% UA in the diet significantly decreased serum MCP-1 levels versus the control group (p=0.016). Surprisingly, at study endpoint (post-tumor), MCP-1 levels with 0.25% UA were unchanged from the pre-tumor value but were not significantly different from the lower post-tumor control value, while MCP-1 levels were significantly lower compared to control in mice receiving 0.05% and 0.10% UA (p=0.013 and 0.037, respectively). In blood collected after 3 weeks on the diets, there was a gradual, dose-dependent increase in serum IGF-1 levels as the UA dose increased; however, it was statistically significant in only the 0.25% UA treatment group. Serum IGF-1 data from study endpoint were not available because of insufficient serum.

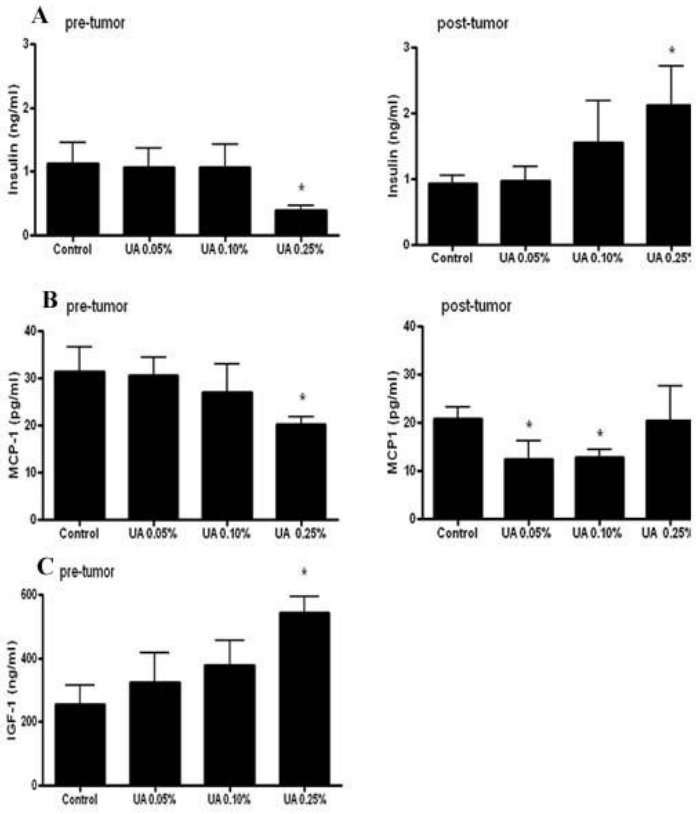


Figure 5.4. UA effects on circulating levels of insulin, MCP-1, and IGF-1.

Serum levels of insulin (A) and MCP-1 (B) after 3 weeks of dietary UA treatment (before tumor injection) and at study endpoint (Week 8) in ovariectomized C57BL/6 mice receiving UA supplementation. Serum levels of IGF-1 (C) were assayed after 3 weeks only. Values represent mean ± SEM from groups (n=9-10/group). *Different letters indicate significant differences ($p<0.05$).

5.4.4 URSOLIC ACID DECREASES AKT ACTIVATION IN MAMMARY TUMORS IN VIVO

Because we saw changes in IGF-1 serum levels, we determined the effect of UA treatment on downstream growth factor signaling pathways in the tumor samples. Akt is a serine/threonine kinase that mediates pro-survival signaling in many tissues, and it is known to be activated via insulin/IGF-1 receptors (193). Receptor level/activation was analyzed by examining IGF-1/insulin receptor (IR) protein expression in tumors from these mice by western blots, and no effect was observed (data not shown). We also analyzed Akt phosphorylation, which is a major downstream effector of both insulin and IGF-I, in the 3 UA treatment groups by western blot. Total Akt protein expression did not change among the treatment groups, but phosphorylated Akt (Ser473) levels decreased in a dose-dependent manner as a result of UA supplementation (**Figure 5.5**). Both 0.10% UA and 0.25% UA treatment groups displayed significantly decreased Akt (Ser473) phosphorylation levels versus control.

The ribosomal protein S6 is one of the downstream targets and main effectors of the mammalian target of rapamycin (mTOR) and is directly involved in translation. Therefore, to confirm downregulation of the Akt pathway by dietary UA supplementation, we determined the phosphorylation status of ribosomal protein S6 (Ser235/236) in tumors from the UA treatment groups and controls by western blot. No changes were observed in total S6 expression; however, a significant decrease in ribosomal protein S6 (Ser265/569) phosphorylation in 0.10% UA and 0.25% treatment groups ($p<0.05$ for both groups) was observed, while 0.05% UA treatment did not affect S6 (Ser235/236) phosphorylation. The effects of UA on Akt and S6 activation were thus very similar. These data strongly suggest a potential Akt/mTOR modulation by UA in MMTV-Wnt-1 mammary tumors.

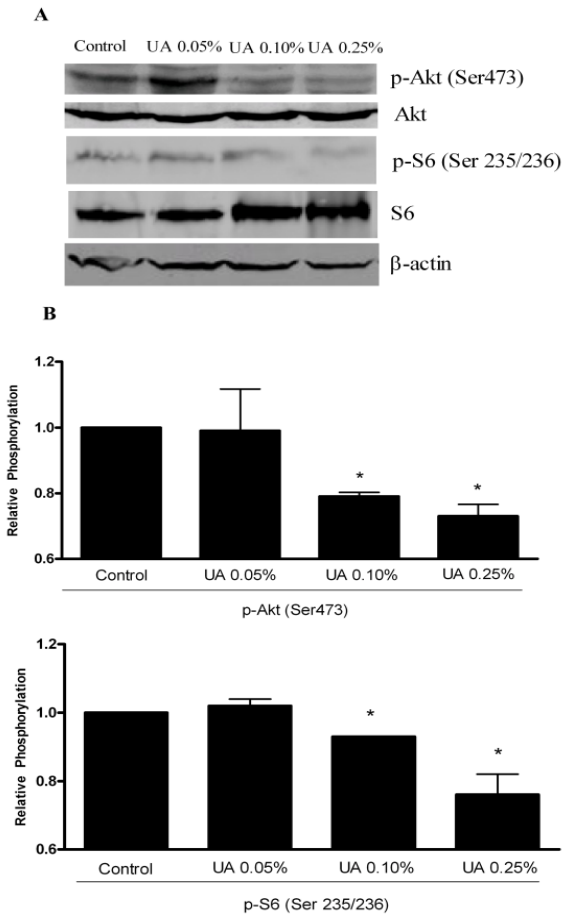


Figure 5.5. Ursolic acid decreases Akt and S6 phosphorylation in MMTV-Wnt-1 mammary tumors.

Western blots were performed on tumor lysates. 0.10% and 0.25% dietary UA significantly decreased Akt (Ser473) phosphorylation and S6 (Ser235/236) phosphorylation relative to the control ($p<0.05$). A representative blot is shown in (A). No changes in total Akt or S6 protein levels were observed. Values represent the mean of triplicate experiments, and data are presented as relative phosphorylation normalized to the control (B). *Different letters indicate significant differences ($p<0.05$).

MAPKs are downstream of growth factor pathways involved in cell growth, survival, and proliferation, and UA has been shown to modulate members in the MAPK transduction pathways, including effects on S6 phosphorylation (98). Therefore, we determined the effects of UA treatment on MAPK p44/42 (Erk1/2) protein expression and phosphorylation/activation by western blot in tumors harvested from control and treated mice. No changes were observed in total MAPK (data not shown). The 0.05% UA treatment caused a significant increase in phospho-MAPK p44/42, but the two other doses (0.10% and 0.25%) decreased MAPK phosphorylation compared to control levels.

Because UA treatment appeared to modulate Akt and MAPK, which play critical roles in controlling cell differentiation, survival, and apoptosis and can influence cell cycle regulators (194), we examined changes in cyclin D1, a cell cycle regulator, and cleaved-caspase-3, a key modulator of apoptosis that is known to regulate proteolytic cleavage in the apoptotic pathway. We found that cyclin D1 was significantly downregulated in tumors from mice receiving 0.10% UA, relative to the controls, while no changes in cyclin D1 were observed in tumors from mice receiving 0.05% and 0.25% UA (**Figure 5.6**). Surprisingly, unlike results seen with IHC (**Figure 5.2**), cleaved-caspase-3 protein expression did not correlate with tumor size or phosphorylation states of Akt (Ser473) or MAPK p44/42 (data not shown).

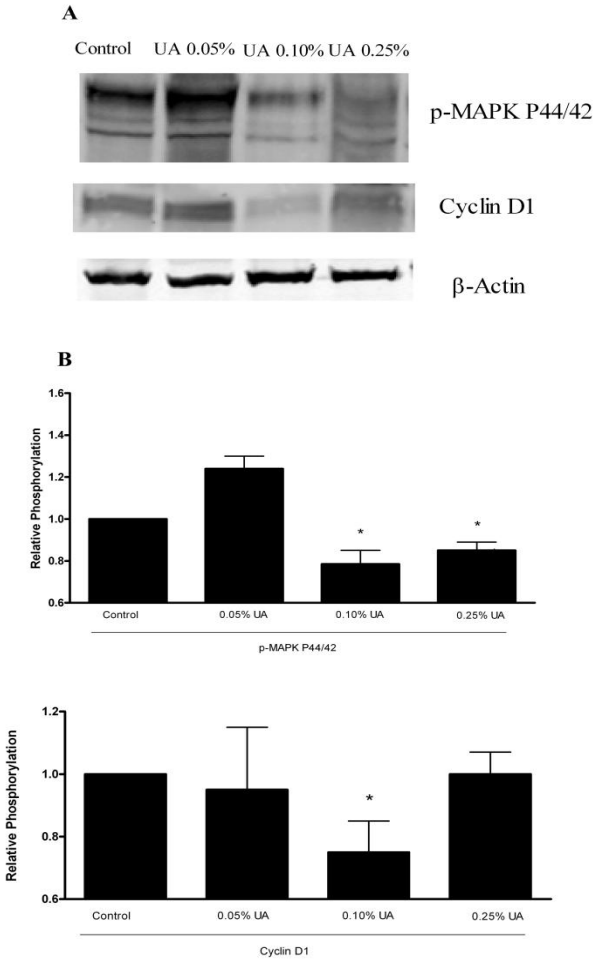


Figure 5.6. UA effects on phsophorylation of MAPK and protein expression of cyclin D1 in MMTV-Wnt-1 mammary tumors.

Western blot analyses were performed on tumor lysates. 0.10% dietary UA decreased phosphorylation of MAPK (p44/42) and protein levels of cyclin D1, while UA 0.25% was effective in decreasing phosphorylation of MAPK (p44/42) only. A representative blot is shown in (A). Values represent the mean of triplicate experiments, and data are presented as relative to the control. Different letters indicate significant differences ($p<0.05$).

5.4.5 URSOLIC ACID INHIBITS PROLIFERATION IN WA4 CELLS IN VITRO

To further elucidate the effect of UA on mammary tumor size, we tested the growth inhibitory effects of UA on WA4 mammary tumor cells in vitro. As shown in **Figure 5.7A**, UA was effective in inhibiting the proliferation of WA4 cells at the two highest concentrations (25 and 50 μM), with almost no cells present at 50 μM after 24 h. As early as 24 h of exposure, cells incubated with 25 and 50 μM UA displayed some morphological characteristics of apoptosis, such as cell shrinkage and the presence of apoptotic bodies.

5.4.6 INHIBITION OF WA4 COLONY FORMATION BY URSOLIC ACID

We used the CFC assay to assess the ability of UA to inhibit tumor colony formation in these cells and determine if UA by itself is sufficient for cell growth inhibition. Compared to control, all UA concentrations decreased the number of tumor cell colonies formed by WA4 cells (**Figure 5.7B**), with the maximum antiproliferative effect, equal to no colony formation, of UA at 50 μM. Few colonies developed from cells incubated with UA at 25 μM. The size of the colonies was also smaller in wells incubated with 25 μM compared to control and 12.5 μM UA (data not shown).

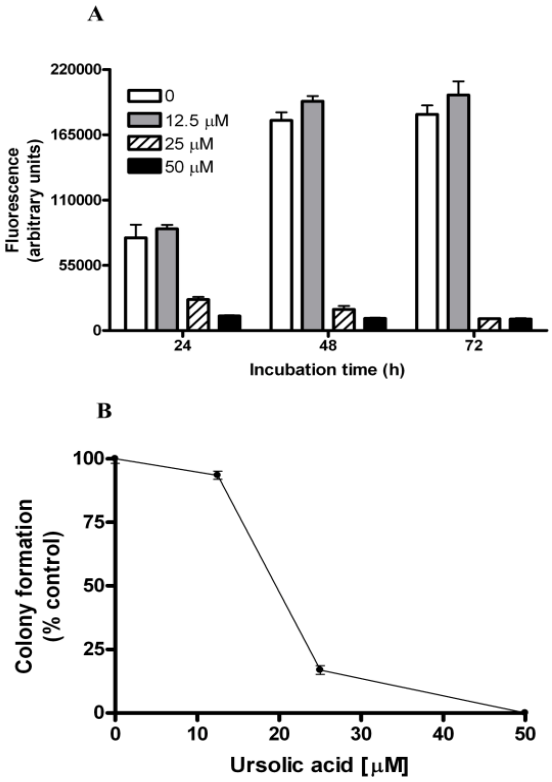


Figure 5.7. UA treatment leads to decreased cell viability and colony formation of WA4 cells in vitro.

UA effect on cell viability was determined by the calcein AM assay (A), in which the fluorescent signal of calcein is proportional to the number of living cells. Cell viability is expressed as percent of the control cells that received 0.1% DMSO (vehicle). Each data point represents the average of six wells. Colony-Forming Cell (CFC) assay (B) shows that UA was effective in inhibiting the ability of single cells to grow and proliferate. Values represent the mean of triplicate experiments. Results are mean ± SEM. Significant differences from control are indicated by *. Significance level is $p<0.05$.

5.4.7 URSOLIC ACID EFFECTS ON CELL CYCLE AND APOPTOSIS IN WA4 CELLS IN VITRO

To determine whether effects on cell cycle progression mediate the growth inhibitory effects of UA on WA4 cells, cell cycle distribution was examined after 24 h of UA treatment by flow cytometry. Compared to the control, UA treatments resulted in a progressive and significant (p=0.03) accumulation of cells in the G1 phase (**Figure 5.8**), demonstrating that UA is a potent cytostatic agent. The lowest UA concentration tested, 12.5 μM, increased the percentage of cells in G1 from 48.40% (control) to 59.61%. This effect was more evident at the highest UA concentrations, 25 μM (65.31%), and 50 μM (69.82%). WA4 cells incubated with 12.5 μM UA also showed an increase in the percent of subdiploid population, with dramatic increases at 25 μM UA and 50 μM UA. This subdiploid population, however, cannot be solely categorized as apoptotic, as it may contain a mixture of necrotic cells and debris as well.

We also evaluated the effect of UA on apoptosis and cell death using the Annexin V-FITC apoptosis assay (**Figure 5.9**). Early-stage apoptotic cells are positive for Annexin V staining after nuclear condensation has started but are not yet permeable to PI. Cells also positive for PI staining are late apoptotic or necrotic cells. The comparison of our results between cells positive for Annexin V and for both Annexin V and PI indicated that the percentage of late apoptotic/necrotic cells was much higher than apoptotic cells (**Figure 5.8**), as the percentage of late apoptotic/necrotic cells increased from 5.74% in the control to 7.48% at 12.5 μM UA, and dramatically increased to 64.9% at 25 μM UA and 75.3% at 50 μM UA. The concentration-dependent increase in the percentage of cells positive for both Annexin-V and PI is an indication of the increased leakiness and membrane disruption that are characteristics of late stages in cell death.

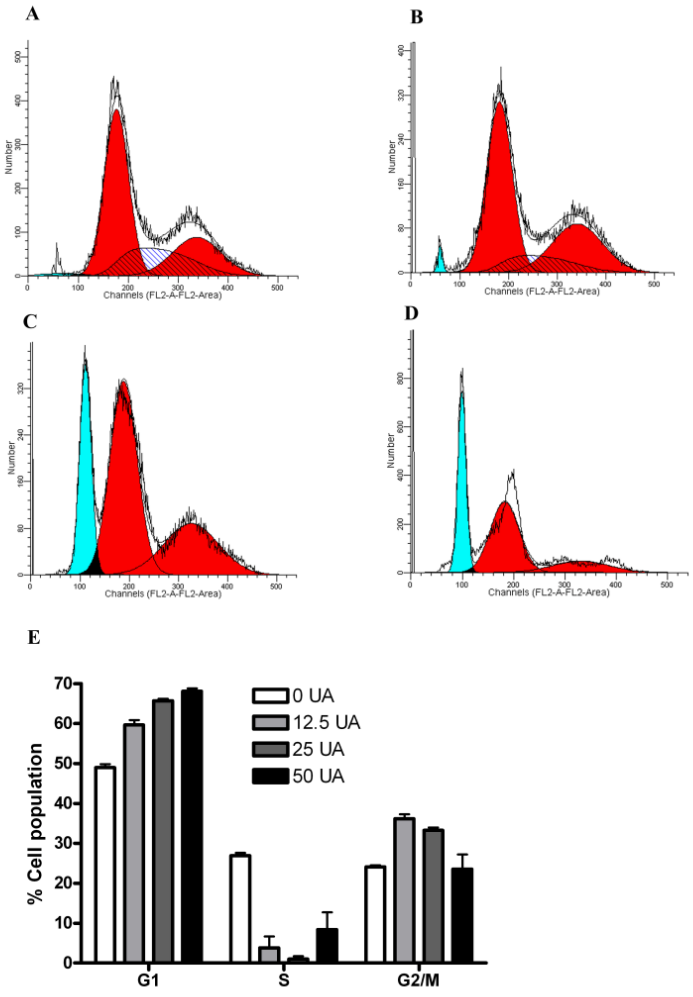


Figure 5.8. UA inhibits cell cycle progression in WA4 cells.

The effects of incubation with UA (0, 12.5, 25, 50 μM) on cell cycle distribution of WA4 cells after 24h are shown in panels A, B, C, and D, respectively. UA caused an inhibition of cell cycle progression with a concentration-dependent increase in the percentage of cells in G1. Cells were fixed with paraformaldehyde and stained with PI, followed by cell cycle distribution analysis by flow cytometry. Data from 50,000 gated cells were collected for each data file. The percentage of cells in each phase of the cell cycle was calculated using ModFit software (E).

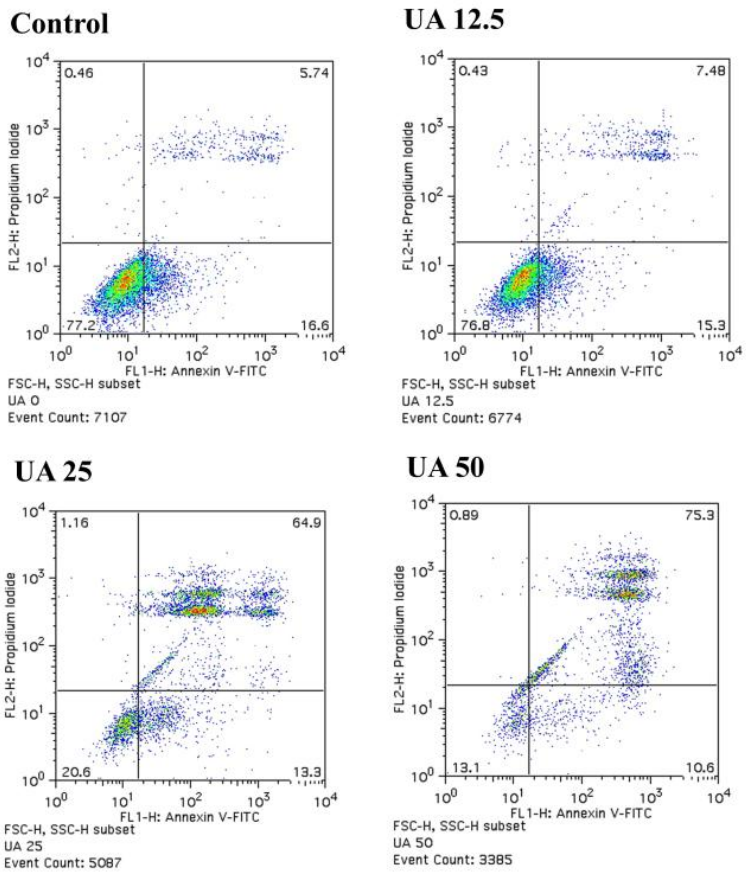


Figure 5.9. UA induces apoptosis in WA4 tumor cells.

Dot plots of Annexin V-FITC/PI flow cytometry (representative data) after 24 h incubation are shown. For each plot, the lower left quadrant shows the percentage of viable cells (negative for both Annexin V and PI). The lower right quadrant represents the percentage of cells undergoing early apoptosis, in which the integrity of the membrane is not disrupted (Annexin V-FITC positive and PI negative) The upper right quadrant contains late apoptotic and necrotic cells (positive for both Annexin V-FITC binding and PI uptake).

5.5 Discussion

In this study we investigated the effects of UA on mammary tumor cells in vivo and in vitro. Our results suggest that UA has anti-tumor activity in a mouse model of postmenopausal breast cancer, and these findings are supported by its inhibitory effects on an MMTV-Wnt-1 mammary tumor cell line in vitro; however, there was no clear dose-response relationship in vivo. Ovariectomized mice were fed diet supplemented with 0.05%, 0.10%, and 0.25% (w/w) UA, injected orthotopically with MMTV-Wnt-1 mammary tumor cells after 3 weeks, and maintained on the diets until the end of the study. While 0.10% and 0.25% dietary UA significantly reduced the size of palpable tumors through day 26 after tumor cell injection, only the 0.10% UA treatment reduced final tumor weight; in the last week of the study the highest dose appeared to promote tumor growth, suggesting that UA may exert dose-dependent opposing effects. In support of this notion, it has been reported that, while UA is able to induce differentiation of teratocarcinoma stem cells and also to inhibit key aspects of angiogenesis and proliferation in endothelial cells, UA stimulates other steps of angiogenesis, including cell invasiveness, within the same system (195, 196). Other bioactive food components have been shown to have differing effects in the same system depending on the level of exposure (197); a well-known example is the soy phytochemical genistein, which in breast cancer cells has been shown to have multiple activities and a biphasic effect on proliferation (198). In our study, it is noteworthy that all doses of UA, but not the control diet, completely inhibited the appearance of a palpable tumor in a subset of mice; however, similar to the effect of UA on final tumor weight, the maximal level of inhibition (40%) was seen with the 0.10% UA treatment.

Triterpenoids, including UA, have been shown to inhibit cellular proliferation (199) and induce apoptosis (180). In the present study UA inhibited proliferation in vivo in a dose-

responsive manner, as assessed by immunohistochemical staining of Ki67 in the tumor tissue. Surprisingly, only 0.10% UA increased immunohistochemical staining of cleaved-caspase-3, a marker of apoptosis, in the tumors; however, this biphasic effect of UA on apoptosis is inversely similar to its inhibition of final tumor weights and may partially account for the lack of a dose response. In WA4 mammary tumor cells in vitro, incubation with UA caused a G1 block in cell cycle traverse in a concentration-dependent manner, with significant accumulation of cells in the G1 phase at all concentrations tested. In addition to this cytostatic activity, UA at higher concentrations was very effective in reducing the number of viable cells, which correlated with the high proportion of late apoptotic/necrotic cells observed, and in inhibiting colony formation.

The effects of UA on cell survival and apoptosis have been shown to be mediated by the PI3K/Akt and MAPK cellular signaling pathways (98). In our study, the two higher doses of UA (0.10% and 0.25%) decreased phophorylation of Akt and of a downstream effector of Akt/mTOR, S6, and also decreased MAPK phosphorylation in tumor tissue. These findings support Akt and MAPK as signaling pathways targeted by UA. However, only the 0.10% dose of UA decreased levels of cyclin D1, a downstream effector and key regulator of the G1/S transition, but this lack of dose response correlated with the inhibitory effects of UA on final tumor weight. Also, stroma and epithelium could contribute differently to tumor growth. Stromal tissue usually shows remodeling of matrix component, while epithelium contains genes associated with mitosis (200). We did not isolated stroma from epithelial tissue for the western blot analyses, and this could explain the lack of agreement with the mammary tumor size.

Elevated circulating insulin and IGF-1 levels are associated with increased risk of breast cancer (201). In our study, consumption of the highest dose of UA (0.25%) for 3 weeks initially lowered serum insulin and increased serum IGF-1 levels, but insulin levels were also elevated at

the end of the experiment when the tumors were collected. UA itself has been shown to act as an insulin-sensitizing agent and insulin receptor activator (202, 203). The elevated levels of these growth-promoting hormones could partially explain the increase in final tumor weights seen with the highest dose of UA; however, the lack of an effect on levels of phosphorylated IGF-1/insulin receptor in the tumors (data not shown) suggests that UA is unlikely to be acting through these receptors, either directly or indirectly through effects on serum hormones.

Inflammation has been shown to be a critical component of breast tumor progression (204), driven in part by cellular stress that leads to increased release of pro-inflammatory chemokines, including MCP-1 (205). Although UA is generally considered to be anti-inflammatory, it may also have pro-inflammatory effects (180). Few in vivo studies have evaluated the effect of UA on inflammatory markers that may influence neoplastic growth. In our study, the highest dose of UA appeared to have an anti-inflammatory effect, as suggested by reduced serum MCP-1 levels after 3 weeks of consumption. However, at the end of the experiment when tumors were collected, the effects of UA on MCP-1 levels were unclear; while the level of serum MCP-1 seen with the highest dose of UA remained unchanged, it was not different from the decreased MCP-1 level in the control mice. Moreover, the two lower doses of UA significantly decreased MCP-1 levels even further.

Considering the lack of dose-response, determination of bioavailability and biodegradation of UA in future studies will be necessary to more efficiently identify effects of UA in particular models systems. We are currently working on validating a new method to extract UA from solid tissues.

In summary, UA displayed anti-tumor activity in a mouse model of postmenopausal breast cancer; all doses tested inhibited development of a palpable tumor in a subset of the mice.

While there was no clear dose response, the intermediate dose of UA, which inhibited development of palpable tumors in 40% of mice and was effective in reducing final tumor weight, also increased staining for cleaved-caspase-3, a marker of apoptosis, and decreased levels of cyclin D1, a proliferation effector, in tumor tissue. In addition, there was a dose-dependent decrease in Ki67 staining, a marker of proliferation; furthermore, the two higher doses of UA decreased activation of the PI3/Akt and MAPK signaling pathways. We also confirmed the cytostatic activity of UA in a mammary cancer cell line derived from the tumor cells tested in vivo. These findings support further consideration of UA as an anti-tumorigenic agent in breast cancer.

Chapter 6: Concluding Remarks

Obesity rates in the US are elevated in most age-categories and unfortunately this trend correlates with the increased risk of several chronic diseases including some cancers. Specifically, risk of breast cancer is increased in adult obese women.

Obesity is characterized by increased levels of adipocyte-related factors including leptin, insulin, and IGF-1. Independently, these factors can activate molecular signaling pathways involved in cell survival and proliferation. The Akt/mTOR signaling pathway can drive breast tumor progression and is activated in response to these factors during obesity.

Considering that the prevalence of obesity is increasing, efforts have been taken to identify mechanism of the obesity-breast cancer connection. The current clinical evidence suggests that weight loss could be an effective approach to decrease breast cancer risk. Here we show that dairy calcium is an effective strategy to decrease postmenopausal weight gain without affecting food intake in C57BL/6 mice. This approach could potentially minimize the risk of mammary cancer.

Although there is some evidence showing that weight loss is protective, there are limited clinical and preclinical studies evaluating the effect of weight loss on postmenopausal breast cancer risk. In addition, these studies have not evaluated metabolic and molecular changes that follow weight loss. In our studies, we looked at the effects of both obesity and former obesity on postmenopausal mammary tumor growth. In our transplanted Wnt-1 mammary tumor model, we found that obesity reversal does not hinder tumor progression or dampen Akt/mTOR signaling in the timeframe studied. However, we found that treatment with RAD001 (10 mg/kg BW), an mTOR inhibitor, effectively decreased tumor growth in the CR and control group (by 81% and 61% respectively), and to a lesser extent in the formerly obese (48%). A higher dose of RAD001

(15 mg/kg BW) was necessary to dramatically decrease tumor growth in the formerly obese group (76%). Our results suggest that RAD001 can overcome the protumorigenic nature of obesity on mammary tumor development through its effects on the mTOR pathway. Our findings raise important considerations in the treatment of breast cancer after weight loss, and suggest that a combination of lifestyle and pharmacologic strategies may be effective for breaking the obesity-breast cancer link.

Further investigation of the "former obesity" effect revealed that of the obesity-related hormones and cytokines we measured, only circulating IGF-1 levels remained elevated (comparable to obese mice). This suggests that recent weight loss is not sufficient to dampen tumor proliferative signals consequent to obesity-associated elevations in circulating IGF-1 and Akt/mTOR signaling. Since IGF-1 production is stimulated by growth hormone (GH), we analyzed GH levels in serum from control, obese, and formerly obese mice and found a trend towards a decrease in GH levels in serum from control, obese, and formerly obese mice. GH secretion may be a secondary effect since its levels are usually reversed after normalization of body weight. However, elevated levels of IGF-1 found in our study seem to be independent of GH secretion (**Figure 6.1**).

More mechanistic studies are needed to evaluate the contribution of obesity-related factors in the progression of postmenopausal breast cancer. Our laboratory is currently in the planning stages of a preclinical study to evaluate the effect of obesity and formerly obesity on response to endocrine therapy for postmenopausal breast cancer. We have preliminary data that indicates a decreased response to aromatase inhibitors in the context of obesity in our mouse model of postmenopausal breast cancer. Results from these studies will help to understand the complexity of the obesity effect on breast cancer.

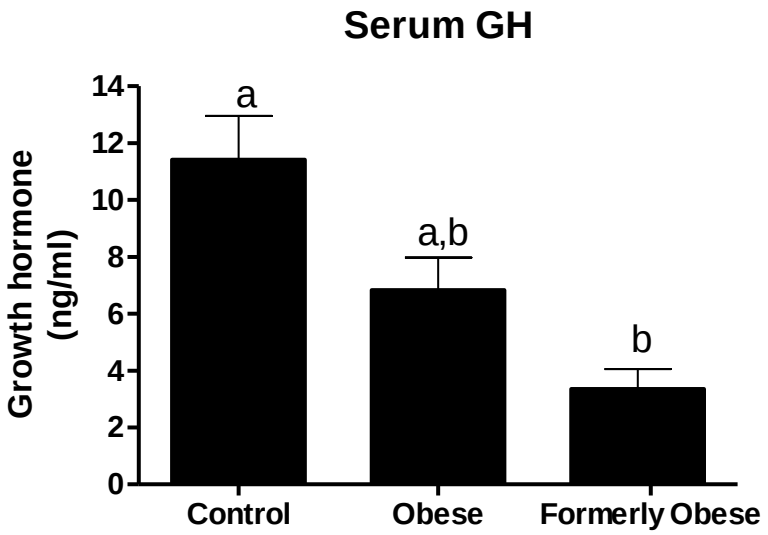


Figure 6.1. Serum levels of growth hormone in C57BL/6 OVX mice.

GH levels were low in obese mice relative to control, however it did not reach significance (p=0.07). Levels in serum from formerly obese were significantly lower relative to control (p=0.002), however were not different from obese (p=0.06).

Since IGF-1 is primarily produced and released by the liver, we assessed expression of IGF-1 and IGF-1R in the liver by RT-PCR We found that only obese mice had elevated IGF-1R mRNA expression in the liver relative to control mice (**Figure 6.2**).

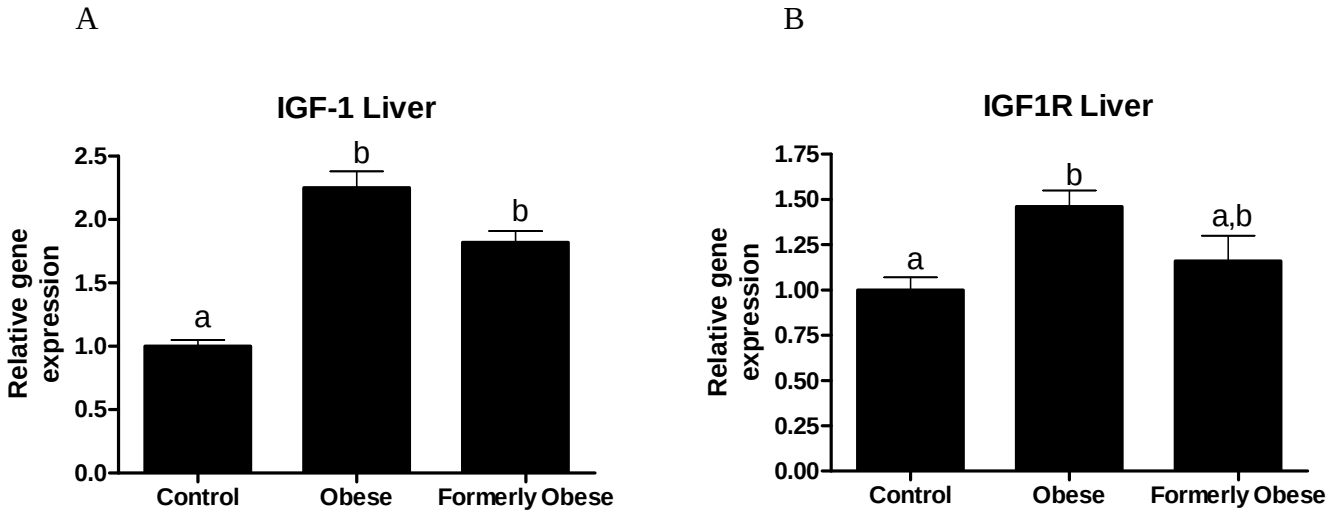


Figure 6.2. IGF-1 and IGF1R gene expression in liver from C57BL/6 OVX mice.

IGF-1 gene expression was higher in livers from obese and formerly obese mice relative to controls ($p<0.05$) (A). Liver from obese mice had increased IGF1R gene expression relative to livers from control animals ($p<0.001$). IGF1R gene expression in the formerly obese mice was not different from control or obese mice.

Given that most breast cancer cases will develop therapeutic resistance, alternative approaches to target increased activation of key signaling pathways will be useful. Natural components have been used for many years in the treatment of many diseases. In the past few years, researchers have been trying to understand mechanisms of action of bioactive food components during tumor cell growth. Here we showed that UA is an effective strategy to inhibit Akt/mTOR signaling and decreased MMTV-Wnt-1 tumor growth. Since UA effect is p53-dependent, future studies in our lab will evaluate UA effect on p53+/- and p53+/+ MMTV-Wn-1 mammary tumor cells.

FUTURE DIRECTIONS

As we previously discussed, obesity effects are detrimental to overall health, particularly increasing the risk for postmenopausal breast cancer. Over the past years, research has shown that alterations in molecular mechanisms as a result of obesity can drive breast cancer growth. Furthermore, the effect of obesity on postmenopausal breast cancer is in part mediated by Akt/mTOR signaling activation in response to increased levels of obesity- associated factors.

As discussed in Chapter 1, weight loss has been associated with beneficial effects on diabetes and insulin resistance among other co-morbid conditions. In addition, body weight reduction is often accompanied by changes in growth factors and mitogenic hormones such as insulin, estrogen, and leptin that are elevated in the obese state. These hormones/growth factors activate signaling pathways that converge at mTOR. However, it is not know if these molecular alterations will return to basal levels following weight loss nor the definite role of weight loss on postmenopausal breast cancer risk remains to be fully elucidated.

In our series of studies, we found that despite normalization of body weight, persistent effects of obesity continue to drive MMTV-Wnt-1 tumor growth in C57BL/6 mice. Here we show that

formerly obese mice continue to have elevated MMTV-Wnt-1 tumor growth that is no different from their obese counterparts. Molecular analysis revealed that Akt/mTOR signaling is equally activated in both obese and formerly obese mice. Furthermore, serum levels of IGF-1 were also equally elevated in both of these groups relative to the control mice and IGF-1 can directly activate Akt/mTOR.

Our studies raise important considerations for postmenopausal breast cancer after recent weight loss. Ideally, future studies would evaluate the temporal relationship between weight loss and risk of postmenopausal mammary cancer. More in particular, determining when circulating IGF-1 is returned to basal levels following weight loss. Furthermore, since IGF-1 can activate Akt/mTOR signaling during obesity, targeted animal studies would aim to evaluate the persistent effect of obesity following weight loss and circulating IGF-1 levels. We found that 4 weeks after changing from the DIO regimen to the control diet, IGF-1 levels were not normalized. Therefore, it would be relevant for future studies to evaluate a longer timeframe in order to see a reduction in IGF-1. In addition, incorporation of a group of inducible liver-specific IGF-1 deficient mice (iLID) to future studies will be ideal to investigate the role of IGF-I when weight loss is an issue. The iLID mouse model is characterized by acute IGF-1 deficiency. The iLID mice express the Cre recombinase under the control of the anti-trypsin 1α promoter in liver, and a single tamoxifen injection results in gene recombination to produce a liver-specific IGF-I deficiency (iLID) (206). Results from these studies will identify a role for IGF-1 in former obesity and MMTV-Wnt-1 tumor growth, and its role driving Akt/mTOR signaling after weight loss. It would also identify likely drug targets, immediately following weight loss, in order to decrease mammary cancer risk.

There is a growing body of evidence supporting the effect of obesity on gene regulation through epigenetic modifications (207). Taken together, our studies argue in favor of more mechanistic studies investigating epigenetic alterations that may occur in response to obesity. These changes can potentially drive the persistent effects of obesity on mammary tumor growth. In addition, analysis of post-translational modifications in adipose tissue of obese, formerly obese, and never-obese mice would lead to a better understanding of genomic changes that may extend the effects of obesity even after normalization of body weight.

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Vita

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